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A CALCIUM DEFICIENCY SYNDROME PRODUCED IN GROWING ANIMALS

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(*Received February 11, 1937*)

INTRODUCTION

The first inorganic element to assume paramount importance in the study of human nutrition was calcium. It has been more extensively investigated than any other, with the result that the literature is filled with studies of this element in its relation to various physiological functions. The object of this paper is to attempt to draw from experimental results conclusions concerning manifestations of tetany in dogs maintained on a diet optimal with respect to vitamin D content, but markedly deficient in calcium. Shelling and Asher ('32) and Templin and Steenbock ('33) stated an inability to produce tetany in rats despite almost complete elimination of calcium from the diet, and depletion of calcium reserves in the body. In view of the inherent differences in stability of the nervous system of various species, an experiment was indicated, using an animal whose reactions differed from those of the rat. No attempt will be made to review the literature on the subject, as there are in existence several comprehensive reviews (Peters and Van Slyke '35, Stewart and Percival '28, Cantarow '31) of this phase of nutritional research.

PROCEDURE

Young dogs were selected weighing approximately five or six pounds. It is a decided advantage to use puppies, as the requirements for calcium are much greater during the period of active growth, thus accentuating the effect of calcium deprivation. The animals were brought into the laboratory and fed a stock powdered ration to accustom them to the conditions under which the experiment would be carried out. At the end of one week they were all placed on the experimental diet, which had the following composition:

	Per cent
Acid washed casein	20.00
Salt mixture No. 60	4.953
Butterfat	8.0
Starch (Argo)	55.05
50% Alc. yeast ext.	100 cc. (equivalent to 10 g. yeast)
Viosterol	15 drops per kilo.
Lard	2.0 added daily to ration

(This diet by analysis showed 30 parts per million of calcium.)

The control diet contained salt mixture #63 replacing salt mixture #60 in the experimental diet. Salt mixture No. 63 is fed at a level of 6.453. The diets were fed ad libitum. The animals were housed in metal cages with wide mesh false bottoms to prevent coprophagy. The composition of these salt mixtures is:

	No. 60	No. 63
KCl	1.000	1.000
$NaHCO_3$	0.700	0.700
$Fe_2(SO_4)_3NH_4)_2SO_4$	0.508	0.508
KH_2PO_4	1.700	1.700
NaCl	0.500	0.500
$MgSO_4$	0.545	0.545
$CaCO_3$	——	1.500

A total of twelve animals was used, the set being divided into four groups of three each. One animal in each group served as a control. Twenty cubic centimeter samples of blood were taken weekly and analyzed. The following constituents were determined (method indicated by references): sodium (serum) (Kerr '26); potassium (serum) (Kerr '26); calcium (serum) (Tisdall '23); inorganic phosphorus (serum) (Fiske and Subbarow '25); magnesium* (serum), the modification by Gittleman and Grayzel of method of Kramer and Tisdall ('21); chlorides (whole blood) (Wilson and Ball '28); fatty acids, (plasma) (Bloor '28); total cholesterol and cholesterol esters (plasma) (Bloor and Knudsen '16); lipoid phosphorus (whole blood) (Benedict and Theis '24); total fat (plasma) calculated as sum of fatty acids and total cholesterol; free cholesterol (plasma), calculated as difference between total cholesterol and cholesterol esters; albumin, fibrinogen and globulin (plasma) (Wu '22); non-protein nitrogen, preformed creatinine and creatine (whole blood) (Folin and Wu '19); sugar (whole blood) (Benedict '31); CO_2 capacity (plasma) (Van Slyke '17); serum bilirubin (Van den Bergh '21); erythrocyte volume (whole blood) (Van Allen '25); uric acid (Benedict '31); urea (Folin and Suedberg '30) and hydrogen ion concentration (Lang '30).

At death, a complete autopsy was performed. The third animal, the control, in each group of three was killed and autopsied when the two experimental animals had succumbed to the deficiency. The four control animals showed no macroscopic pathology, as would be expected from their normal behavior and normal blood chemistry while living.

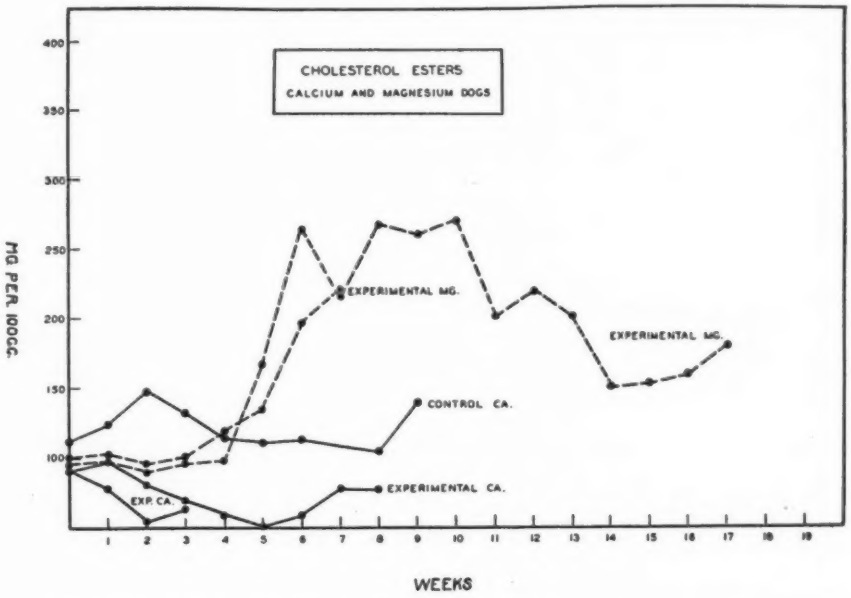
Daily food consumption of controls and experimental animals was recorded. No significant variations were noted until the experimental animals became moribund. This period of approximately two or three days before death was marked by almost complete cessation of food intake by the experimental animals.

RESULTS

Chemical.—The following changes were observed in the blood picture. The serum calcium remained within normal limits, 9.8 mg. per 100 cc. of the serum being the lowest value observed in experimental animals. Serum calcium values of the controls averaged 12.0 mg. per 100 cc. A blood sample taken during a tetanic seizure in one instance showed a normal value of 11.1 mg. per 100 cc. of serum. The inorganic phosphorus content of the serum dropped from an average normal value in the controls of 7.9 mg. per 100 cc. of serum to 5.3 mg. per 100 cc. of serum in the experimental animals.

* After calcium has been removed as oxalate, the magnesium is precipitated as $MgNH_4PO_46H_2O$ which is determined by method of Fiske and Subbarow.

The lipid picture is, briefly, a decrease in the total fat with concurrent decrease in fatty acids and a decrease in total cholesterol, the decrease in total cholesterol being largely due to a decrease in the cholesterol esters. There was no change in the lipoid phosphorus content of the serum. The cholesterol ester changes are illustrated by the graph below.



A peculiar shift in chloride content of the blood serum was noted. Whereas the total chloride content of whole blood remained unchanged, the serum chlorides increased, indicating a shift of chloride from the formed elements of the lobod into the serum. The increase in serum chlorides amounted to approximately 5 per cent. This increase is significant, as the method (Wilson and Ball '28) used is accurate to within 1 per cent.

A decrease in pH with the accompanying decrease in the carbon dioxide combining power of the blood was observed. Controls showed a CO_2 combining power of 50 volumes per cent, while in experimental animals the value decreased to 35 volumes per cent.

Blood taken from several of these animals in a moribund condition showed a great increase in non-protein nitrogen (200 per cent), while the glucose content decreased to 50 per cent of its normal value.

Symptomatology.—Animals placed on the low calcium diet, despite the apparently normal serum calcium values, exhibit a remarkable succession of symptoms which terminate in death. Within a month manifestations of the deficiency are evident, the prone position maintained by the dogs being due either to

muscular degeneration or to pain manifested in an erect posture. The condition was primarily unilateral, as evidenced by the fact that the animals lie almost entirely on the left hind leg, which is curled under the body. Response to tactile stimuli in the thoracic cord region was normal, but in the lumbar and sacral cord region responses to such stimuli indicated a hypersensitivity. During this stage in the course of the deficiency, manifestations of gastro-intestinal disturbances are noted, diarrhea being a constant symptom. Bloody stools were not seen. One animal, mature when placed on the diet, maintained an erect position for several months. This dog in which halisteresis was not so marked showed lateral curvatures in the long bones.

The stage of hypersensitivity is succeeded by a period during which there are no manifestations of sensitivity. The animals, still in a prone posture, place the legs in positions which indicate that the joints are dislocated. The articular capsuls and ligaments seem relaxed, permitting a separation of the bones. This condition was also noted at autopsy. Uniformly, the hind legs showed abnormality before the fore legs. Weight decreases were observed during this stage.

In the final stages the animals showed mild tetanic seizures. At the onset of the convulsion the entire body, rigid from a tonic contraction, is thrown into a characteristic posture. The head is drawn back, and the extremities are stiffly extended. Palpation showed the muscles to be in a tonic spasm. Respiration ceases during the attack and returns with subsidence of the tonic convulsion. With muscular relaxation clonic convulsions set in, with the animal alternately flexing and extending its extremities. Abnormalities in respiration are noted at this time. The inspirations seem normal but prolonged, so that the thorax is ballooned. The expirations occur with explosive violence, causing a peculiar rattling in the throat similar to laryngismus stridulus. There is drooling of regurgitated stomach contents from the mouth. The syndrome invariably has a fatal conclusion.

Three manifestations characterize the syndrome: the position of the animals during the first stage, the hypersensitivity of the lower cord region, and the tonic-clonic convulsions.

Four gross pathological findings were pronounced. Hemorrhages were observed throughout the body, in the viscera, in the muscular tissues, in the bones, and even into the body cavities. The hemorrhages did not seem due to trauma or erosion, but rather to injury of the capillary endothelium. Coagulation time was prolonged, from three to ten minutes, which might bear some relation to the extensive hemorrhages. The osteoporosis observed has been frequently reported in animals on low calcium diets. Due to the halisteresis, the bones could be bent completely so that the epiphysis at the distal end would touch the epiphysis at the proximal end and not break the bone. The relaxation of the articular capsule and ligaments has previously been mentioned. A separation of the bones of the articulations was seen. The inflamed, hemorrhagic gastric and intestinal mucosa with occasional ulceration was pathognomonic of the gastro-intestinal upsets observed.

DISCUSSION

The deleterious effects of calcium deficient diets on growing rats have been discussed by McCollum and co-workers. They have also demonstrated the beneficial effects of vitamin D in partially protecting against the calcium deprivation. Shelling and Asher ('32) report their experimental diet as being much lower in calcium than those used by McCollum. Analysis showed 0.012 gm. per cent, which is 120 parts per million. They report that "the serum calcium does not fall to tetanic levels in growing rats reared on low calcium-optimal phosphorus diets over long periods of time." Templin and Steenbock ('33) fed a diet containing 0.058 per cent of calcium, approximately 500 parts per million, and failed to observe tetany in rats, even when the diet contained no vitamin D. In the presence of vitamin D, they report maintenance of serum calcium at 10.9 mg. per 100 cc. The diet used in this experiment contained 30 parts per million of calcium and is therefore materially lower in this element than diets used by other investigators. On this diet, serum calcium was maintained within normal values as previously reported (Shelling and Asher '32, Templin and Steenbock '33). The development of a typical syndrome terminating in tonic-clonic convulsions and death, despite the normal serum calcium levels in these animals, has not previously been observed. Several conditions have been reported in which manifestations of tetany were observed with a normal serum calcium value: gastric (McCann '18, Hastings et al '21, McCallum et al '20, Tisdall '22), bicarbonate (Howland and Marriott '18, Harrop '19, Davis et al '20, Greenwald '22), and magnesium tetany (Kruse '32). However, none of these conditions is at all similar to that described here, as in the cases reported the magnesium tetany differs in the decreased magnesium content of the serum.

Gastric tetany is the result of an obstruction in the stomach, and is characterized by a reduced chloride content of the blood. These features distinguish it from the condition described here. Bicarbonate tetany, being characterized by an alkaline condition of the blood, can be differentiated from the other types. In this study we are dealing with tetany in the presence of a tendency toward an acid condition of the blood. Here, again, the generalization that alkalosis underlies most forms of tetany is aside from the fact. Although the symptomatology of this condition seems very similar to that of other tetanic conditions, excepting the magnesium syndrome with its vasodilatation, the blood chemistry establishes it as unique. We wish to suggest that the condition may be associated with changes in the distribution of serum calcium from an ionic (Cameron and Moorhouse '25, Cushny '19, Greenberg and Gunther '30, Nicholas '32, Newhausen and Pincus '23, Thompson and Collip '32) to a bound form. Stepp and Gyorgy ('17) have suggested that it is not hypocalcemia but lowering of the calcium ions which is responsible for the tetanic reaction, and the evidence here presented would support this contention.

The terminal rise in non-protein nitrogen is a change familiar in the premortal stage of certain moribund conditions. The blood lipid values observed in magnesium deficiency are indicative of a disturbance in fat metabolism which throws

the burden of maintaining life upon protein metabolism. The increased non-protein nitrogen then represents the end stage of impaired nutrition. This interpretation would seem to explain the observed terminal rise in non-protein nitrogen and the abnormal blood lipid picture in calcium deficiency. The typical picture of blood lipids during magnesium deprivation shows normal fat, high total cholesterol, high cholesterol esters and low fatty acids, all in the plasma, and normal lecithin in whole blood (Kruse et al '33). The essential point in which magnesium and cal ium deficiencies differ in the abnormal lipid picture is in the increased cholesterol esters in the magnesium deficiency and the decreased cholesterol esters in the calcium deficiency. This is illustrated in the graph. The opposite effect shown by a deficiency of calcium and of magnesium on the cholesterol ester content of serum might be considered as another manifestation of the "ion antagonism" exhibited between these elements. Granting this hypothesis, a diet deficient in both calcium and magnesium should show a blood lipid picture intermediate between the two. The validity of the conclusion that an ion antagonism effe.t is being manifested in these deficiencies has been strengthened by the work of Day, Kruse and McCollum ('35) who observed in dogs on a diet deficient in both calcium and magnesium a blood lipid picture intermediate between those of calcium or magnesium deficiencies. The work of Epstein ('31) must, however, be considered as a possible explanation of the decreased cholesterol ester values observed in the calcium deficiency. He noted a decreased synthesis of cholesterol esters in conditions characterized by dysfunction of the liver. A knowledge of the extent of liver damage in the animals on low calcium diets awaits histological examination.

SUMMARY

Dogs fed a diet low in calcium (containing 30 parts per million of calcium) maintain their serum calcium level within normal limits throughout the entire course of the deficiency. Despite this apparent normality of the serum calcium, the animals showed a characteristic syndrome terminating in tonic-clonic convulsions. Abnormalities in blood chemistry established the condition as unique.

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A COMPARISON OF NUTRIENT SALT SOLUTIONS FOR THE CULTIVATION OF EXCISED TOMATO ROOTS

By

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A study planned to determine if any of a number of salt solutions chosen from the literature of plant and animal nutrition may be superior for isolated tomato roots to that used in previous studies.

The nutrient solutions employed in all studies of excised roots to date have been modifications of Knop solution ('65), with considerable changes in the proportions and absolute concentrations of the constituent salts but without important changes in the particular salts used. Kotte ('22), Malyschev ('32), and Gautheret ('35) used Knop solution, Robbins ('22) the closely related Pfeffer solution. White's modification of Uspenski solution ('34) more closely resembles Pfeffer solution than it does the original formula of Uspenski and Uspenskaia ('25). Formulae for many other nutrient salt solutions have been published in the literature of plant nutrition. These range in complexity from the 3-salt solution of Shive ('15) to the 8–salt solution of Trelease and Trelease ('33) and the even more complex "A-Z" solution of Hoagland and Snyder ('33). The possibility that some solution other than those previously used might prove more satisfactory for excised roots led to the testing of a considerable number of such solutions.

For this purpose, formulae of 14 standard solutions representing a wide range of compositions were chosen from the plant nutrition literature. Three solutions commonly used in animal nutrition studies (McCollum et al., '28; Ringer, '86; Tyrode, '10) were added for comparison. The concentrations of all constituents of these solutions were recalculated so that the total molar concentration of each solution should equal that of the modified Uspenski solution used in all of the author's work ('34), which will hereafter be referred to as "standard solution." This is essentially a Pfeffer solution with reduced phosphate content, having a salt concentration of 2.7 millimols. The salt proportions of all the plant-nutrient solutions were kept unchanged. Because of the use of NaCl in considerable concentration in Ringer ('86) and Tyrode ('10) solutions, as an osmotic agent, an exact proportionate transformation of these solutions to the molar concentration of the standard solution would have left the concentrations of cations other than Na much below the minimum requirements for plants. For this reason the NaCl concentration in these solutions was reduced by an amount 10 times as great as the other salts. This gave a final Na:K ratio approaching that of the few plant-nutrient solutions which regularly contain sodium (Schimper, '90; Sachs, '60; Schreiner and Skinner, '10; Mazé, '19). When the source and concentration of iron to be added was not specifically stated in the original reference, $Fe_2(SO_4)_3$ at the concentration

TABLE 1

No.	Salt	Grams in 1 liter of a 0.01 M solution		Standard	Knop*	Pfeffer*	Schimper*	Tottingham*	H.W.& P.*	Crone*	Sachs*	Schreiner & Skinner*	Shive	Trelease& Trelease†	Mazé††	Marchal & Marchal	Beijerinck**	McCollum et al.	Ringer***	Tyrode***
		Anhydrous salt	Hydrate	Milliliters of a 0.01 M solution in 1 liter of nutrient = concentration in millimols multiplied by 100																
1	$CaCl_2$	1.11												66			25		15	20
2	$CaCO_3$	1.00													160					
3	$CaSO_4$	1.36	1.54							30	50					60				
4	$Ca(NO_3)_2$	1.64	2.36	60	130	115	100	85	115				35							
5	$Ca_3(PO_4)_2$	3.10								15	45									
6	$CaH_4(PO_4)_2$	2.34	2.98						25			5						35		
7	Ca-lactate	2.18	3.03															10		
8	KCl	0.74		90		30			65										20	20
9	KNO_3	1.01		80	55	50	40	30		175	110			110						
10	K_2SO_4	1.74										25				50				
11	K_2HPO_4	1.74					25							2	15		35	90		
12	KH_2PO_4	1.36		10	40	35		90					125	40	20					
13	K-citrate	3.06	3.24											1						
14	NaCl	0.58					70				20							35	205	110
15	$NaNO_3$	0.85										240			64					
16	$NaHCO_3$	0.84																	30	105
17	NaH_2PO_4	1.20																40		5
18	$MgCl_2$	0.95	2.03																	10
19	$MgSO_4$	1.20	2.46	30	45	40	35	65	65	40	45		110	40	10	70	25	50		
20	NH_4NO_3	0.80														20	185			
21	$(NH_4)_2SO_4$	1.32												10						
22	$(NH_4)_2HPO_4$	1.32														65				
23	Fe_2SO_4	1.52	2.64											1						
24	$Fe_2(SO_4)_3$	4.00		0.6	0.6	0.6	0.6	0.6	0.6		0.6	0.6	0.6		1.3	5.0	0.6		0.6	0.6
25	$Fe_3(PO_4)_2$	3.58								10										
26	Fe-citrate	2.45	3.00															10		
Total molar concentration				2.706	2.706	2.706	2.706	2.706	2.706	2.700	2.706	2.706	2.706	2.700	2.703	2.700	2.706	2.700	2.706	2.706
Bibliography number				'34	'65	'00	'90	'14	'07	'02	'60	'10	'15	'33	'19	'13	'93	'28	'86	'10

*Formulae taken from Tottingham ('14).
** Formula taken from Moore ('03).
*** Formulae taken from Bayliss ('24).
† Only the common salts of Trelease and Trelease's formula are included. The "accessory salts" have been omitted.
†† Only the common salts of formula are included. The "accessory salts" have been omitted.

regularly used in the standard solution, 0.006 millimol, was added. The salt concentrations of the solutions tested are given in table 1. Two per cent of sucrose and 0.01 per cent of yeast extract were included in all nutrients ('32). All roots used were derived from the standardized clone C ('34) and were selected for uniformity. All cultures were treated uniformly except for the variations in salt constituents under examination. Since it was not possible to test all solutions simultaneously, the results were calculated as percentages of the growth index in the standard solution kept as control in each series of cultures. Ten cultures were grown in each solution for one week and each series was repeated 3 times. All cultures were measured daily and the results averaged. The numerical indices given are thus the means of the results from 30 cultures grown at 3 different times.

TABLE 2

Solution	Growth expressed as percentages of the control										pH*
	Days							Total	Passage 2	Passage 3 (control)	
	1	2	3	4	5	6	7				
1. Standard	100	100	100	100	100	100	100	100	100	100	5.5
2. Shive	92	94	**(116)**	**(109)**	**(109)**	96	83	99	78	—	4.5
3. Tottingham	93	94	80	76	86	70	75	82	73	—	5.8
4. Knop	**(121)**	73	83	83	78	74	71	81	87	—	4.7
5. Schimper	74	87	85	79	75	66	83	78	76	—	4.8
6. Pfeffer	91	88	73	74	83	51	58	72	74	—	4.9
7. H. W. & P.	55	72	62	54	79	66	65	65	98	—	4.9
8. S. & S.	55	62	55	61	65	56	55	58	49	—	4.7
9. T. & T.	62	60	45	42	57	38	46	49	66	—	4.8
10. Crone	71	38	11	13	10	10	12	20	30	69	6.1
11. Mazé	86	30	8	8	1	1	1	14	0	0	7.2
12. Ringer	64	30	8	6	1	3	1	12	0	12	7.3
13. Beijerinck	55	25	11	9	3	1	1	12	0	16	6.4
14. McC., R. & B.	74	19	4	5	1	0	4	11	0	0	6.2
15. M. & M.	40	27	10	5	3	1	3	10	0	0	6.7
16. Tyrode	50	19	7	6	3	1	1	10	0	0	7.7
17. Sachs	43	25	6	4	3	1	1	9	0	9	6.1

* Determined with glass-electrode potentiometer.

The results of the tests are presented in table 2. While an unmodified Knop solution gave a higher growth index on the first day of the passage than did the control, and Shive solution gave higher indices on the 3rd, 4th, and 5th days, the *total* growth index for the entire passage was lower than that of the control in every other nutrient. In none of the solutions studied, with the exception of Shive solution, did the growth index approach that of the control closely enough to leave any doubt of the superiority of the latter as a nutrient for isolated tomato roots.

All of the first 9 solutions in table 2 gave fairly good results. It is probable that cultures *could* be maintained in them indefinitely, though at a low level of increment. These solutions were placed in group 1 in classifying the different

solutions used. The last 8 solutions, constituting group 2, gave very poor results and the demarcation between the groups was quite sharp. At the end of one week each culture was transferred to a solution similar to that used in the first passage for one week and then all cultures in group 2 were returned to the standard solution to test if they had been injured in the experimental solutions. In the second passage the order of growth percentages within groups 1 and 2 differed somewhat. Shive solution dropped from second to fourth place. But all of the first 9 solutions again maintained growth 50 per cent of that in the control or better, while the last 8 solutions again gave little or no growth. In the third passage, carried out in the standard solution, only those cultures taken from Crone solution ('02) recovered satisfactorily, although single roots transferred from the solutions of Beijerinck ('93), Ringer ('86), and Sachs ('60) showed some recovery. The solutions of Mazé ('19), Marchal and Marchal ('13), Sachs ('60), McCollum, Rask, and Becker ('28), Ringer ('86), and Tyrode ('10) were not merely inadequate to support growth but were actually injurious to isolated roots.

An exact analysis of all of the factors responsible for the differences in behavior of these various solutions will require a much more extensive study than has been possible in the time available for this work. An examination of the results presented in table 2 in the light of the formulae in table 1 shows, however, one striking correlation. All solutions in group 1 were relatively acid, all of them having reactions below pH 6.0. All solutions in group 2 were much less acid, having reactions regularly above pH 6.0. Reaction of the solution would thus appear to be of paramount importance for the growth of tomato roots.

Other factors are of less importance. McCollum solution, for example, falling in group 2 is characterized by a very high phosphate content, yet a high phosphate content is not universally objectionable, for Shive solution, standing second only to the standard solution in group 1, has 14 times the phosphate content of the latter. Of the 9 solutions falling in group 1, only 3 depart widely from the Knop solution type. These are the solution of Trelease and Trelease ('33) in which $CaCl_2$ replaces the $Ca(NO_3)_2$ of Knop solution (the addition of traces of K_2HPO_4 and $(NH_4)_2HPO_4$ are probably without much significance), that of Hartwell, Wheeler, and Pember ('07) in which $CaH_4(PO_4)_2$ replaces the KH_2PO_4 of Knop solution, and that of Schreiner and Skinner ('10). This last solution bears little resemblance to any of the other satisfactory solutions, and is anomalous in that it completely lacks magnesium in any form. It will be recalled, however, that earlier work of the author ('33) cast doubt on the importance of magnesium for the development of isolated chlorophyll-free plant tissues. This solution is clearly worthy of closer study, in spite of its low position (8th place) in the order of usable solutions. The marked superiority of the Knop type of solution in general is evident, and a study of the formulae in group 1 shows that within the Knop-Pfeffer-Shive type of formula a rather wide range of salt proportions is possible. As was shown elsewhere ('33), this is much more true of anion than of cation proportions.

Beijerinck solution ('93) has long been ranked as an excellent nutrient for

algae, fungi, and sometimes for seed plants. Its ranking in 13th place in a series of 17, in the present study, was thus surprising. Since, however, this solution if unadjusted is relatively much less acid than are the satisfactory solutions and an acid reaction appears to be important for tomato roots, the effect of readjustment of the solution reaction was tested on Beijerinck solution and 3 other solutions. For this purpose 4 solutions, having unadjusted pH's of 4.5

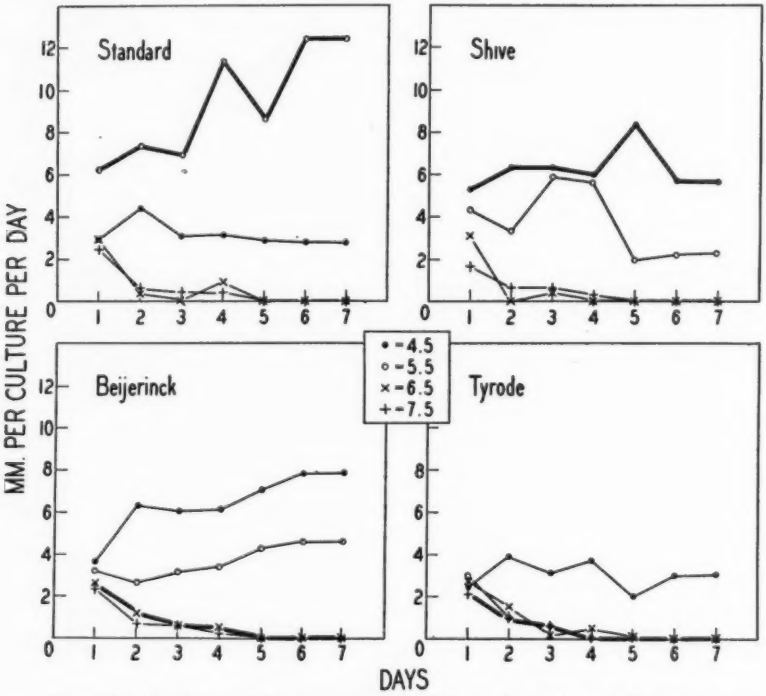

FIG. 1. Graphs showing daily increment rates of isolated tomato roots during one passage (7 days) in four different nutrient solutions, each at four different hydrogen-ion concentrations. Solid circles = pH 4.5, open circles = pH 5.5, crosses = pH 6.5, and plus signs = pH 7.5. The heavy line in each graph represents the increment rates at the unadjusted pH characteristic of the particular solution shown.

(Shive), 5.5 (standard), 6.4 (Beijerinck), and 7.7 (Tyrode), were chosen and aliquots of each were adjusted to the hydrogen-ion concentrations characteristic of each other solution. Ten cultures were made at each reaction in each solution. The standard solution at pH = 5.5 served as control. The growth curves of these 16 sets of cultures over a single passage are shown in figure 1. At pH 4.5 Shive solution and Beijerinck solution were both superior to the standard solution at that pH, maintaining mean growth levels of approximately 6 mm. per culture per day. This is a fairly satisfactory growth index, though not equal to that of the unadjusted control. The standard solution and Tyrode solution gave about

equal results, with indices around 4 mm. per culture per day. At pH 5.5 the standard solution was markedly superior to the others with a mean index of 9.3, while roots in Shive solution, Beijerinck solution, and Tyrode solution were poorer than they had been at pH = 4.5. At pH = 6.5 and 7.5, cultures grew very poorly in all four solutions.

The significance of these results will be clear if the unadjusted pH values of the four solutions (table 2) are remembered. The standard solution and Shive solution gave their best results at their unadjusted pH values, being injured by any adjustment tested. Beijerinck solution and Tyrode solution, which have reactions above pH 6.0 when unadjusted, were greatly improved by acidification. Beijerinck solution, which is of negligible value at its unadjusted pH of 6.5, became as effective as was Shive solution and almost as effective as the control when adjusted to Shive solution's normal pH of 4.5. Tyrode solution, which in some of its many modifications is extensively used in the cultivation of animal tissues, was entirely worthless for tomato roots if kept at its unadjusted pH, but if acidified to pH = 4.5 became fairly satisfactory, though the improvement was not sufficient to justify its extensive use with plant tissues. The initial failure of Beijerinck solution to give satisfactory results was thus not due to its constituents as such, but to the pH equilibrium brought about by the particular salt combination used. The degree of acidity of the nutrient, as was suggested by the results shown in table 2, was a matter of prime importance. A distinctly acid medium is required by tomato roots. This is the more interesting in view of the neutral or alkaline reaction required by animal tissues.

Since the best results obtained in this series of tests were with the standard solution unadjusted, and since, when other solutions gave satisfactory results they did so at the same reaction or a more acid one than that normal for the standard solution, the author will continue to use this solution in future work.

SUMMARY

The relative efficiencies of 17 salt solutions as nutrients for isolated tomato roots were tested. The standard solution previously used in this laboratory gave the most satisfactory results, with Shive solution second. All satisfactory solutions had reactions below pH 6.0, all unsatisfactory ones had reactions above pH 6.0. Beijerinck and Tyrode solutions were greatly improved by acidification. An acid reaction thus appears to be necessary for satisfactory growth of isolated tomato roots.

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DEVELOPMENTAL GROWTH AND NUCLEIC ACID COMPONENTS—V. CYTOSINE

By

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This is a report of an experimental evaluation of Cytosine in terms of the developmental growth of Obelia geniculata. There is evidence presented which indicates this component of nucleic acid is significantly a participant in some process essential for expression of differentiation.

This report completes the survey of the pyrimidine components of nucleic acid with respect to their influence singly on growth of *Obelia geniculata*.

Details of description and procedure are referred to elsewhere (Hammett and Schlumberger '37). Suffice it to state here that the cytosine as prepared from sheep thymus by one of us (T.F.L.) had the chemical and physical properties essential for establishment of its identity and purity. Stock solutions containing 0.056 gram of the compound in 100 cc. fresh sea-water provided daily the aliquots from which test culture solutions were made.

Seventy experiments were run with close to 37,000 animals, half of which were exposed to cytosine in concentrations ranging from M/400,000 to M/6250. Tests and controls were run simultaneously under like conditions of temperature, illumination, and pH. Four changes into fresh sea-water were made during the 24 hour course of each experiment. The data are given in Table 1.

RESULTS

There is hint that cytosine may be slightly toxic to Obelia in M/6250 concentration. The evidence is that 13 of the 34 growth expressions were less in test than in control material and but seven were greater; while in the lower M/12,500 concentration, test growth was greater in ten and less in nine. The shift from a greater to a lesser expression of catabolism in the progression from the lower to the higher concentration is supportive (Hammett '36a.)

Although changes expressive of metabolic reaction were slight a trend to greater than usual catabolism was exhibited in the surely non-toxic concentrations, and regression seems to show a tendency towards retardation. Conversely any deviation of maintenance was towards strengthening. There was no evidence of specific dynamic effect.

The analysis will be confined to the M/400,000–M/12,500 concentration range.

Initiation was retarded in the presence of cytosine. Thus—new organism production in the tests was less than control in 20 and greater in but ten of the 42 expressions given by the six sets of experiments; by concentrations it tended to be less in four and greater in but one of the six; and when the data

are combined—test is less than control in four and greater in none of the seven potentialities for activity expression.

Test proliferation expression inclined towards enhancement. Thus—it was greater than control in 12 and less in nine of the 48 expressions given by the six sets of experiments; by concentrations it tended to be greater in three and less in but one of the six; and when the data are combined—test is greater than control in three and less in but one of the eight potentialities for activity expression. The extent of deviation is, however, but slight.

Test differentiation tended to be increased. Thus—it was greater than control in 24 and less in but 16 of the 60 expressions given by the six sets of experiments; by concentrations it tended to be greater in three and less in but one of the six; and when the data are combined—test is greater in four and less in two of the ten potentialities for activity expression.

Organization was essentially unaffected. Thus—in the tests it was less than control in 14 and greater in 13 of the 54 expressions afforded by the six sets of experiments; by concentrations it tended to be less in two and greater in one; and when the data are combined—test was greater than control in none and less in but one of the nine potentialities for activity expression.

DISCUSSION

It has been pretty generally reported that cytosine may be deaminized to uracil by the intermediary metabolism of a wide variety of organisms ranging from yeast and B. coli, through dogs to man (Hahn '25, '26, Cerecedo '27, '31, Emerson '30, Boivin '30). It has been found that proliferation expression is enhanced in the presence of uracil (Hammett '36b). Further it is reported that uracil is in some way associated with sulfur metabolism (10:11) and it has been demonstrated that the sulfhydryl group is a naturally occurring and essential chemical stimulus to cell increase in number (Hammett '29).

All these several data suggest that the trend to proliferation enhancement exhibited in the presence of cytosine may derive from uracil and the influence thereof on the reduced-oxidized sulfur ratio.

Now it has been noted that the proliferation increase in the cytosine cultures was of low order. And it has been consistently reported that cytosine as such is pretty fairly stable when exposed to the processes of living. It is excreted unchanged in considerable part when fed to various animals (Boivin, '30, Mendel '10, Wilson '23) and the evidence indicates it is thoroughly metabolized when presented in nucleoside combination (Wilson '23, Deuel '24). Perhaps this then is the basis for low proliferation increase—for the stability of the compound would imply meagre or delayed uracil production—the assumed factor for proliferation in these experiments. Conversely the data are supportive of the idea that cytosine influence on proliferation is chiefly indirect as a source of uracil.

Objection to this interpretation might be taken from the fact that whereas uracil acts to forward initiation, cytosine acts to retard; and whereas differentiation tends towards retardation in uracil cultures, it is distinctly accelerated in

TABLE 1

TREND OF TEST DEVIATION FROM CONTROL WITH RESPECT TO DEVELOPMENTAL AND METABOLIC ACTIVITY IN *CYTOSINE* CULTURES. ALSO THE NUMBER OF EXPERIMENTS, COLONIES, HYDRANTHS AND GONOPHORES AT THE STATED CONCENTRATIONS

M/1000×	400		200		100		50		25		12.5		6.25	
No. Experiments	10		10		10		10		10		10		10	
	Cont	Test	Cont	Test	Cont	Test	Cont	Test	Cont	Test	Cont	Test	Cont	Test
No. Colonies	150	150	150	150	150	150	150	150	150	150	150	150	150	150
No. Hydranths	2575	2573	2598	2594	2625	2627	2690	2687	2592	2597	2602	2590	2684	2684
No. Gonophores	125	123	90	81	77	72	112	106	126	117	98	91	220	234
						Growth								
	+	− =	+	− =	+	− =	+	− =	+	− =	+	− =	+	− =
Initiation	1	4 2	1	6 0	2	2 3	4	2 1	1	3 3	1	3 3	2	4 1
Proliferation	1	0 7	0	4 4	3	1 4	2	0 6	3	2 3	3	2 3	2	2 4
Differentiation	3	5 2	4	3 3	6	1 3	2	3 5	5	2 3	4	2 4	2	5 3
Organization	3	2 4	3	1 5	1	4 4	1	3 5	2	2 5	3	2 4	1	2 5
						Metabolism by Single Expression								
Maintenance	5	1 4	4	6 0	4	4 2	7	3 0	5	3 2	6	4 0	4	3 3
Catabolism	3	3 4	5	4 1	5	4 1	5	3 2	7	2 1	6	4 0	4	6 0
						Metabolism by Combined Expression								
	Cont	Test	Cont	Test	Cont	Test	Cont	Test	Cont	Test	Cont	Test	Cont	Test
Regression	48.2	44.4	37.3	38.8	43.1	43.4	41.0	39.0	38.2	36.0	31.2	31.2	31.8	31.9
Catabolism	85.2	85.9	86.6	87.5	79.6	81.2	84.8	84.9	83.6	89.3	82.8	83.2	84.8	83.3
						Increase in Percentage Regression								
	−7.9		4.0		−0.4		−4.9		−5.8		0.0		0.3	

the cytosine. But—if because of cytosine stability uracil production is slight or delayed, the amount or the rate of production could well have been inadequate to produce expression of these characterising reactions. More complete analysis will be reserved for a later communication.

The differentiation forwarding effect of cytosine is as yet untranslatable. It may be expression of specific participation and again it may be expression of reaction to a dicarboxylic by-product (Hammett and Lavine '37). This matter, too, will be discussed at a later session.

The initiation retarding effect accompanies the introduction of an NH_2 group into the pyrimidine molecule. Whether this can be traced down as specific to the group has yet to be determined. Decision must await completion of the survey and evaluation of each participant in terms of the whole.

What these cytosine data do show, however,—and that right definitely—is that the compound produces an effect upon obelia development which is individual, and distinct from that produced by any of the other naturally occurring components of living substance so far studied.

SUMMARY AND CONCLUSION

From 70 experiments with close do 37,000 animals (*Obelia geniculata*) half of which were exposed to cytosine in concentrations ranging from M/400,000 to M/6250 it appears that the compound accelerates expression of differentiation and retards that of initiation.

Proliferation tends to be slightly better than usual, but organization is unaffected.

The compound may be toxic at M/6250 but only slightly.

In its presence catabolism may be somewhat enhanced. There is no evidence of specific dynamic effect and indeed regression inclines towards retardation.

The conclusion is that cytosine has it own influence upon developmental growth in general and that it is of particular significance to some process associated with differentiation.

These studies were made at The Marine Experimental Station of the Institute, North Truro, Massachusetts, with aid from Miss A. Bein of Philadelphia, and the technical assistance of Misses Chatalbash and Elliott, and Mr. Hans Schlumberger to all of whom we are indeed grateful.

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TIME OF DETERMINATION OF HAIR PIGMENTS IN THE MOUSE*

By

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Genetics and experimental embryology deal with closely related problems. The phenomenal development of these sciences has been rather independent and there is a marked paucity of work correlating them. An important reason for the scarcity of correlation studies is that forms such as the amphibia which are excellent embryological material are not particularly suited for genetical investigations. Laboratory mammals, on the other hand, possess many useful mutations for such investigations but embryological experiments involving transplantation from one embryo to another are difficult in all mammals.

Since 1923 Nicholas has attacked problems of experimental embryology in mammals with considerable success. Transplantation of an albino eye from a thirteen day rat embryo into a fifteen day host embryo was performed by Nicholas ('34). By the thirteenth day of development the structure of the eye and the pigmentation of its retina are differentiated, however, so that no information as to the time of determination of structure or pigment resulted.

Trotter and Dawson ('32) rotated blocks of skin on new-born rats and found in this way that hair slope is determined by the factors of tension effective at the time of development of the hair follicle, which takes place to a large extent at about this time. Here, direction of hair slope is determined by a group of purely mechanical factors.

Mention should be made of the important studies recently published by Waterman ('36) and other investigators concerning the capacities for independent self-differentiation exhibited by isolated rabbit and rat primordia transplanted to the omental bursa and kidney of rabbits.

The use of genetic mutations in problems of mammalian experimental embryology is a logical step in bringing about the coordination of these two fields which are separable only for the convenience of the experimenter.

This report is concerned with the time at which the coat pattern is determined in mouse embryos. Three different gene mutations, white-bellied-agouti, A^w, dominant spotting, W, and black and tan, a^t, produce differences in pigmentation in the hair follicles which express the three distinct coat patterns. There are no pigmented granules in the developing hair follicles of newly born mice so the color of the coat has not yet differentiated. At birth the skin is thin and relatively transparent until pigment granules are formed in the hair follicles. From grafts it was found that the pigmentation pattern is already determined at birth in each of the three mutations. Two other characters which promise

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to be most interesting ("piebald" in mice and "hooded" in rats) have not yet been studied because stocks of the proper genetic constitution have been derived but recently. This paper will present only the facts that concern the mutation, black-and-tan.

The mutation, black-and-tan, was described by Dunn ('28) as an allele of the agouti series. Adult black-and-tan mice have a coat which is sharply differentiated with regard to dorsal and ventral surfaces. The hairs of the back are heavily pigmented while those of the belly are cream-colored or white. This gene is dominant to all other members of the agouti series in the manifestation of the light belly part of the character complex; the back may be any color such as blue, brown or black, whereas the belly is always some shade of cream or tan.

Dunn states that, "The contrast between the pigmentation of the back and belly which is most marked in the agouti types and in black-and-tan, but which occurs also in black and in brown, probably does not imply a differential effect of these allelomorphs on the dorsal and ventral pigmentation, but is more probably due to a regional differentiation of the tissues of back and belly whereby one surface reacts more strongly than the other to general pigment-forming agencies. Since the back and belly show differences in the rate of development of both fur and pigment this may well be the clue to the physiological interpretation."

The epidermis and the hair follicles develop from the ectoderm. Therefore, according to Dunn's interpretation, the black-and-tan pattern is probably determined at the time dorsal ectoderm becomes different from ventral ectoderm. The stage of development during which this differentiation occurs can be determined by transplanting both dorsal and ventral ectoderm to new sites (from dorsal to ventral positions and *vice versa*) at progressively earlier stages. If the transplant develops the pigment characteristic of the region it came from we may say that the distinction between dorsal and ventral ectoderm had occurred prior to the operation.

We assume that before there is a distinction between dorsal and ventral surfaces the ectoderm is potentially capable of giving rise to either black or tan pigmentation. By way of explanation it may be stated that cells taken from an albino embryo and grown on a colored animal will produce only albino tissue regardless of where they are placed. Cells from an albino never possess potentialities for any pigment formation. In cases such as black-and-tan in mice the fertilized egg produces cells some of which will give rise to the black pigmentation and others to the tan pigmentation. Our problem is to find the time of the dichotomy after which the cells no longer possess potentialities for formation of both pigmentations but have been limited to an ability to produce one or the other.

METHODS AND RESULTS

The general plan has been to mate a black-and-tan male with his sisters. When one of the sisters gives birth to a litter, embryos are removed from a second sister and pieces of embryonic skin placed on the new-born young but in a different location. Embryonic ventral skin was always placed on the dorsal

surface of the new-born host and embryonic dorsal ectoderm on the ventral surface of the host.

It is preferable that the parents be not only full brothers and sisters, but also members of a single line which has been inbred for three or four generations in order to insure homozygosity for those genes which allow successful heteroplastic grafts. Strong ('36) has shown that tissue grafts between two distinct inbred strains of mice (N and CBA) are uniformly unsuccessful whereas grafts between the hybrids of the two strains and between the hybrid and the parent strains are successful in a large proportion of cases. On the other hand, highly inbred animals are not desirable, first because they usually lack the vigor necessary for host and donor in transplantation experiments, and secondly because we wish to make our matings in such a way that hosts and donors will have a different pigmentation so that we can always be sure that the growing tissue is actually from the donor and not regeneration of host tissues. (Non-specialized epidermis "regenerates" readily.) This "balance" between the proper degree of homozygosity and heterozygosity is essential for work in mammalian experimental embryology. Workers with amphibia apparently have fewer troubles of this sort. Grafts may be made between amphibian species which are differently pigmented, while in mice grafts do not become successfully incorporated as a part of the host unless both host and donor are of the same species and also of very similar genotypes.

The following are details of a typical experiment:

Five young were born to ♀ 87 on the thirtieth of January and were operated upon that day. The five young were etherized, one at a time, and from each a piece of ventral skin about 3 m.m. square was removed from the chest over the middle sternal cartilage. An embryo was then removed by Caesarian section from a sister of ♀ 87 and a piece of dorsal skin placed in the excised area of the new-born animal. The embryonic epidermis was then covered by a small piece of cellophane, thus enclosing the transplanted block in a moist cell. A piece of adhesive tape one half inch long and one quarter inch wide was then placed over the wound and adhesion insured by firm pressure, care being taken not to press the underlying graft. The graft will be incorporated within forty-eight hours and the adhesive tape with attached cellophane comes off within 48–72 hours after it is applied.

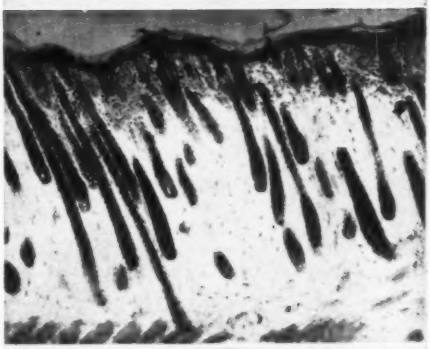
The embryo which was removed by section was measured and then fixed in Allen's modification of Bouin's fluid and sections were made in order to show the histological differentiation at the time the graft was taken. The embryos remaining in the mother will continue their development unharmed if the

FIG. 1. Photomicrograph of a section through the skin of the 17–18 day old embryo (1.8 cm. in length) which acted as donor to the new born ♂ 246. A. The outer layers of the epidermis. B. Malpighian layer or *stratum germinativum* (one cell layer). C. Derma.

FIG. 2. Photomicrograph of a section through the skin of a six-day-old mouse. The differentiation of pigmented hairs has been completed and the hairs are in process of eruption.

FIG. 3. Photograph of the hairs developed by a graft of the ventral skin of an 18 day old black-and-tan embryo onto the back of an ordinary black host.

1



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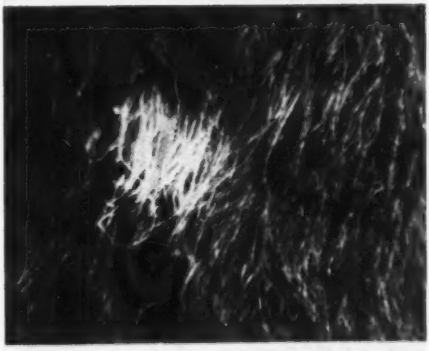

3

FIGS. 1–3

Caesarian operation has been properly performed and if only one or two embryos were removed. In this particular case five normal young were born seventy-two hours after two embryos had been removed. This allows us to calculate the age of the embryos removed, by counting back from the time of birth. As the gestation period is from twenty to twenty-one days the embryos of this experiment were from seventeen to eighteen days of age. Figure 1 shows the histological picture of the skin of the 17–18 day embryo which acted as donor to the new-born ♂ 246. This animal (♂ 246) had, by ten days after birth, developed a good coat which was "blue" dorsally and "tan" ventrally. A large darkly pigmented graft was growing in the center of the chest; four days later it had developed black hairs just as would have been the case if the transplant had been left on the embryo.

Figure 3 shows a host (♀ 359) and the hairs produced by the graft. This graft was the converse of that in the previous case (♂ 246), and of considerable significance. The host, ♀ 359, was an ordinary black animal, but when ventral skin from an eighteen day black-and-tan embryo was grown on her dorsal surface it differentiated "tan" as it would have done if left intact on the embryonic donor; that is, it differentiated "tan" even though on a non black-and-tan animal.

We may, therefore, conclude that once there is a dorsi-ventral *determination* of pigmentation, *differentiation* may then proceed according to this determination regardless of where we place the graft (provided the position is sufficiently favorable for the hair to grow at all). Further, the genotype of the host has no influence so long as it allows compatible growth of the graft. Pigmentation of the host has no effect on pigmentation of the graft if the graft was "determined" before transplantation. Embryonic tissue placed on albinos (♂ 108 and ♀ 108) differentiated black hair on the albinos as it would have done if left on the donor animal.

A brief description of the successful grafts is given in Table 1. Grafts which failed to become incorporated are not recorded here as they gave no information of significance for the problem of determination.

TABLE 1

RESULTS OF SOME OF THE GRAFTS INVOLVING THE MUTATION BLACK-AND-TAN. ALL DONORS GENETICALLY BLACK-AND-TAN

Calculated age of embryo used as donor	Length of donor in cm.	Source of graft	Constitution of host	Position of graft on host	Hair differentiated by graft on host
21 days	2.8	dorsal	albino	chest	black, wavy
			albino	chest	black, wavy
18.5 days	2.0	dorsal	black-and-tan	chest	black
			brown-and-tan	chest	black
			brown-and-tan	chest	black
18.0 days	1.9	ventral	pure black	dorsal	tan
17.5 days	1.8	dorsal	blue-and-tan	chest	black

It may be seen that in each of the tabulated cases the future pigmentation of the embryonic tissue had been determined at the time of transplantation.

It is not surprising that pigmentation was already determined in the oldest embryos. The experiment with the youngest which measure 1.8 cm. is of more interest. The host was ♂ 246 of the experiment described in detail. As the sections of this embryo show (see Figure 1) the skin is composed of two layers. The inner layer (the derma) consists of three to six rows of irregularly placed cells. The outer layer of the skin (the epidermis) is composed of three or four rows of cells; the innermost row of these lies next to the derma and is known as the Malpighian layer or *stratum germinativum*. In the embryo of Figure 1 the Malpighian layer is composed of one row of regularly placed large cells of uniform size. This row is most distinct and shows many clear mitoses. It gives rise to the hair follicles later in development. The hair follicles produce the pigment granules.

As the hair follicles are to arise from this single row of cells (the Malpighian layer), it is this row of cells which was already determined at the time the graft was placed on the new-born ♂ 246. To repeat, the Malpighian layer is a single row of cells absolutely the same dorsally as ventrally. Yet this single row of cells is already determined so that its descendants in the dorsal half of the animal will produce hairs with black pigmentation and its descendants in the ventral half of the animal will produce hairs with tan pigmentation.

The color of the pigment to be formed in the future hair follicles is determined at least as early as the 17–18 day stage of embryonic development. Actual pigment granules do not appear until the twenty-first or twenty-second day. During this span of four days there is a doubling of the body size and extensive development of sense organs and other ectodermal derivatives.

At the 17-day stage the epidermis shows a lack of specialization which is in sharp contrast to that shown at six days after birth (see Figure 2, ♂ 360) when differentiation of pigmented hairs has just been completed and the hairs are in the process of eruption. At the 17-day stage the skin is only from nine to twelve cells thick (both derma and epidermis) and of a loose, slimy, texture which makes transplantation difficult.

We hope to transplant "skin" by including underlying mesoderm in the graft, at the 13 to 15 day stages. Embryological sections of 13 day embryos show the epidermis to be a single row of ectodermal cells. Even the Malpighian layer has not yet differentiated in embryos so young.

We might expect to find this single layer of epidermis already determined in regard to dorsal and ventral pigmentation, for the present experiments show that determination has taken place when the epidermis is but three or four cells thick and while the Malpighian layer consists of but a single row of cells. At least two speculations may be advanced. The single row of cells forming the Malpighian layer has been determined because of differences in underlying dorsal or ventral tissues at the time the Malpighian layer differentiates (14–15 days). Or, determination for pigmentation may take place as soon as the dorsal

surface of the embryo is differentiated from its ventral surface; that is, not later than the end of gastrulation.

One fact stands forth clearly. If the black-and-tan case is representative, embryological determination takes place long before histological differentiation may be observed. When a small difference in pigmentation, such as the black- and tan-case, is determined at such an early date, surely organs with vital functions and involving several primary germ layers in their development must be determined early in gastrulation or considerably before. Such seems to be the case in the amphibia.

DISCUSSION

Experiments with color patterns *not* having a primary symmetry such as the dorsi-ventral pattern of black-and-tan are now necessary, to show whether (in the case of "piebald" for instance) determination is later than it is for the black-and-tan pattern. If this is found to be the case it would permit us to study the causes of determination of those patterns which are determined late enough in development to allow the use of transplantation and tissue-culture technique.

Adopting the suggestion of Dunn ('28) we may say that the black-and-tan basic pattern is present in all normal mice since the back and belly of non-mutant mice show differences in the rate of development of both hair follicles and pigment. When the black-and-tan gene is present the normal dorsi-ventral pattern is much more conspicuous because of the greater contrast between pigmentation of back and of belly. The question then follows: "Are patterns such as piebald in mice and hooded in rats also present in "non-mutant animals?" Such is probably the case, to some extent at least, as genetic modifiers of color pattern have been shown to exist in wild strains of mice which manifest no pattern. These modifiers determine, in part at least, the pattern that will be exhibited if the piebald gene is introduced into the wild stock.

It is hoped that further applications of the techniques presented in this paper will provide answers to some of these questions.

SUMMARY

Some of the possibilities for future study of the time of determination of known gene mutations of the mouse are pointed out.

Three innovations in technique are:

(1) Pieces of embryonic tissue are grown as heteroplastic grafts on new-born young.

(2) Mammalian embryologists find it necessary to have host and donor genetically related for successful transplantation. A genetic "balance" is suggested which insures sufficient homozygosity to permit compatibility between host and graft but at the same time to leave sufficient heterozygosity so that the hairs coming from the graft will have a different pigmentation from those of the host; identification of the graft is thus certain.

(3) Only one or two embryos are removed from the mother leaving the remainder to be born at the normal time. The relative age of the embryo removed

may then be calculated. With calculated age, observed length in centimeters, and histological picture of the embryo experiments may be arranged in their proper sequence with considerable certainty.

With the aid of this technique it was found that the determination of the pigmentation pattern of a gene mutation (black-and-tan) was at a very early stage in the development of the epidermis. It was found that the epidermis, which will later give rise to hair follicles and within them the pigment granules, is already determined with regard to pigmentation when it consists of only three or four layers of cells and its Malpighian layer consists of a single row of histologically identical cells. Descendants of this single row of Malpighian cells will later form the hair follicles within which appear the pigment granules (black on the dorsal surface of the animal and "tan" on the ventral).

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INTESTINAL LENGTH AND BODY WEIGHT IN THE ADULT ALBINO MOUSE

By

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(Received April 6, 1937)

The intestines of adult mice were measured and the figures subjected to a statistical analysis. Evaluation of these data disclosed a relationship between body weight and intestinal length which appears to have escaped attention heretofore.

TECHNIQUE OF MEASUREMENTS

Autopsy was performed within a few minutes after decapitation. The abdomen was opened and the rectum was cut across deep in the small pelvis. The intestine was freed of mesentery with the aid of fine scissors and excised at the pylorus. The intestinal tube, hanging down from its rectal end, was placed upon a suitably graduated glass plate. The lengths of (a) small intestine from pylorus to ileo-caecal valve, (b) large intestine from the ileo-caecal valve to the rectal cut, and (c) total intestine (by addition of (a) and (b)) were ascertained; the caecum was not measured.

EVALUATION

The intestinal measurements of 144 male albino mice of 14 to 30 grams of body weight, from the same stock colony, were collected. The findings reported here with reference to this group were confirmed on another series of 300 mice with a more limited range of weight. Animals of the same weight (± 0.5 grams) were grouped. Each body weight group consisted of 4 to 17 animals (except for the 14, 15, and 16 gram groups which were composed of 1, 3, and 2 animals respectively). The average lengths of the small, large and total intestine of each group are plotted in the accompanying chart (Figure 1). The smoothed average curves* in the chart represent the intestine lengths as a function of the body weight. The total measurements are summarized in tabular form (Table 1).

RESULTS

The intestinal length showed no increase at all concomitant with the high increase in body weight (cf. Figure 1). Actually there was a slight decrease in the length of the intestinal components with increasing body weight. The coefficients of intestinal length per gram body weight, when graphically charted in regard to body weight groups, fit a parabolic curve; e.g., those of small intestine length decrease from ca. 2.6 cm. in the lightest (14 to 17 gram) to ca. 1.3 cm. in the heaviest animals (29 to 30 grams).

In other words, the intestinal length of adult mice was found constant (ca. 48 cm. for total intestinal length, ca. 40 cm. for small intestine, ca. 8 cm. for

* Procured by graphical calculation from the original values of each group.

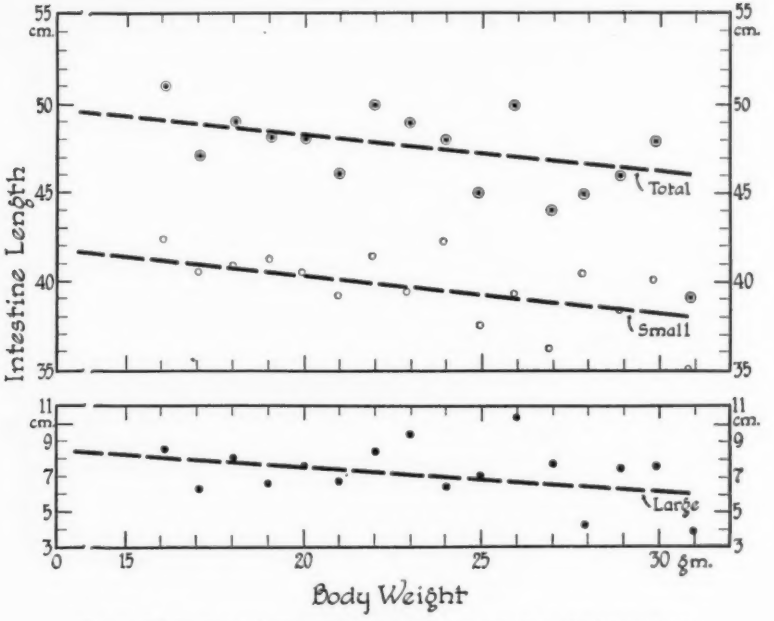

FIG. 1. Relation of intestine length (ordinate) and body weight (abscissa).

large intestine) and independent of body weight. Since body weight can be equated with age (with variations which are of no decisive importance for the present considerations), these observations strongly suggest that after puberty

TABLE 1
SUMMARY OF MEASUREMENTS MADE ON ADULT MICE
(144 Observations)

	Maximum Value	Minimum Value	Total Range	Arithmetic Mean	Standard Deviation	Relative Deviation from the Mean (per cent)
Body weight	30 gm.	14 gm.	16 gm.	23.7 gm.	±4.03 gm.	±36
Length of small intestine	50 cm.	28 cm.	22 cm.	39.9 cm.	±4.74 cm.	±28
Length of large intestine	10 cm.	4 cm.	6 cm.	7.9 cm.	±1.19 cm.	±43
Length of total intestine	58 cm.	35 cm.	23 cm.	47.5 cm.	±5.40 cm.	±32

no further growth is demonstrable in intestinal length. The slight decrease in length with increasing body weight may not only be explained by a disproportionate deposit of fat, but also by a loss of extensibility and elasticity of the intestinal tissue in older animals.*

* The general expression for all the curves determined by the present measurements, representing their type more precisely than an outright constant, is:

These observations provide a standard of reference for intestinal length of the adult mouse which can be utilized whenever the natural spread can be neglected.

The constancy of intestinal length after puberty as illustrated by the present measurements in the mouse is not entirely unexpected. Some observations in the human (Beneke, 1880, Debele 1900, Scammon '23) point to the same direction: the ratio of intestinal length to body weight decreases from 8.3 in the new-born to 5.0 in the adult, and the absolute length of intestine in the adult is only twice that of the new-born. However, we were unable to find any direct confirmation of our observations in the literature with respect either to the mouse or to other species of animals. Because of the lack of comparable figures, an attempt was made to compare the figures here presented with those obtained by Riddle and Nussman from the intestines of doves (Riddle and Nussmann, '33). The procedure used in making these measurements in the dove differs from that described above only in small details. Tearing and active stretching was carefully avoided in our procedure. Although this might result in comparatively lower figures for the mouse intestine, nevertheless, the total average figures indicate that, in relation to the body weight, the intestine of the mouse is approximately 10 times longer than that of the dove. A still more important difference is seen in the intestinal length/body weight relationship. If we plot the average lengths published by Riddle and Nussmann as we have done for our own figures we find that the lengths of intestine in adult doves vary with the body weight.

SUMMARY

The lengths of small, large, and total intestine were measured in adult male albino mice. There is no increase in any of these divisions with increase in the body weight although the body weight ranges from 14 to 30 grams. The conclusion drawn from these observations is that after puberty the length of the intestinal canal remains practically constant irrespective of further body growth.

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Intestinal length (cm.) $= K - k$ (body weight),

where K and k are empirically determined constants. K has moderately (about 10%) higher values than the three constants given above, and may possibly represent the length of an "ideal" intestine of optimal extensibility. k is a fraction (about 0.2), and may possibly represent the circumstances enumerated which are responsible for the slight decrease of intestinal length with increasing body weight or age.

THE GROWTH OF PINEAPPLE PLANTS IN COMPLETE WATER CULTURES WITH EITHER AMMONIA OR NITRATE SALTS*

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(*Received June 15, 1937*)

INTRODUCTION

The growth of pineapple plants *Ananas comosus* Merr. under field conditions when plotted against time forms a sigmoid curve in accordance with the results of various investigators (2, 3). The weight values of such plants as obtained periodically are subject to certain inherent errors resulting from variations either in their initial weight or in their performance after planting under field conditions.

However, more accurate values can be obtained with plants grown in water culture as such errors can be greatly eliminated because the same plants are weighed periodically.

HISTORICAL

Investigations, in recent years, on the growth phenomena of various species of both lower and higher forms of plants and animals have shown that the periodically increasing mass (weight or volume) of an organism can be expressed mathematically by certain well known formulae. Robertson (8) has well summarized our knowledge of the subject up to 1923. Since then other contributions have considerably enriched our knowledge of the physicochemical reactions of growth.

With respect to plants the data of Reed and Holland (4) and Reed (5, 6, 7) show that quite satisfactory results can be obtained using the formulae of autocatalysis either as originally employed by Robertson (8) or as suggested, according to Reed (7), by Bray and Davis (1).

EXPERIMENTAL

Two sets of plants, grown in water cultures one with ammonium and the other with nitrate as sources of nitrogen, were employed for these studies. The plants were propagated from slips (vegetative organs produced at the apex of vestigial fruits), which had been cured for 14 days in the greenhouse. After trimming, that is, removal of the old and injured sections of the leaves and stem they weighed 105 ± 15 grams. On January 10, 1936 they were set in water cultures which were maintained at pH 5.0 ± 0.4 and their first roots emerged about January 25 to 30. Plant weights, obtained at times as indicated in tables

* Published with the approval of the Director as Technical Paper No. 103 of the Pineapple Experiment Station, University of Hawaii.

TABLE 1

GROWTH OF *Ananas comosus* MERR. IN AMMONIUM NUTRIENT SOLUTIONS AND COMPARISON OF CALCULATED AND OBSERVED VALUES OF x FROM LOG $x/2631-x=0.0066\ (t-313)$

t	x Obs.	x/A	$K(t-t_1)$	$t-t_1$	K	$K(t-t_1)$ Calc.	x/A Calc.	x Calc	Divergence	
									Grams	%
42	36	0.014	−1.848	271	0.00682	−1.79	0.016	42	+ 6	17
71	107	0.041	−1.369	242	0.00566	−1.60	0.024	63	− 44	41
108	168	0.064	−1.165	205	0.00569	−1.36	0.042	111	− 57	34
156	271	0.102	−0.945	157	0.00602	−1.02	0.087	229	− 42	15.5
192	412	0.157	−0.730	121	0.00603	−0.800	0.137	360	− 52	12.6
227	574	0.218	−0.555	86	0.00645	−0.568	0.213	561	− 13	2.5
253	794	0.302	−0.364	60	0.00607	−0.396	0.287	755	− 39	4.9
282	1011	0.384	−0.205	31	0.00662	−0.205	0.384	1011	0	0
316	1346	0.512	+0.021	3	0.00700	+0.020	0.511	1346	0	0
352	1684	0.640	0.250	39	0.00642	0.258	0.645	1696	+ 12	0.7
365	1821	0.692	0.352	52	0.00677	0.343	0.688	1810	− 11	0.6
416	2246	0.852	0.760	103	0.00738	0.680	0.827	2176	− 70	3.1
445	2474	0.942	1.211	132	0.00917	0.870	0.881	2320	−154	6.2
478	2631	1.000		165		1.100	0.927	2441	−190	7.2

1 and 2, were made after the individual plant had been removed from its container and its root system had drained thoroughly. The experiments were discontinued when the plants had fruits which had made about one-fourth of their final weights. From the combined weights of the plant and fruit that of the latter was subtracted to obtain the former. The fruits were produced when the

TABLE 2

GROWTH OF *Ananas comosus* MERR. IN NITRATE NUTRIENT SOLUTIONS AND COMPARISON OF CALCULATED AND OBSERVED VALUES OF x FROM LOG $x/2547-x=0.00666\ (t-304)$

t	x Obs.	x/A	$K(t-t_1)$	$t-t_1$	K	$K(t-t_1)$ Calc.	x/A Calc.	x Calc.	Divergence	
									Grams	%
0	0									
42	37	0.0145	−1.83	−262	0.00700	−1.75	0.0175	44	+ 7	19.0
71	112	0.0440	−1.337	−233	0.00575	−1.55	0.0270	69	− 43	38.4
108	161	0.0633	−1.170	−196	0.00597	−1.30	0.0470	120	− 41	24.8
156	282	0.1110	−0.904	−148	0.00610	−0.986	0.094	240	− 42	14.9
192	433	0.170	−0.689	−112	0.00616	−0.746	0.152	388	− 55	12.7
227	619	0.243	−0.493	−77	0.00640	−0.513	0.235	600	− 19	3.1
253	832	0.327	−0.313	−51	0.00617	−0.340	0.313	798	− 34	4.1
282	1052	0.414	−0.151	−22	0.00686	−0.147	0.416	1060	+ 12	1.1
316	1392	0.547	+0.082	+12	0.00684	+0.080	0.546	1390	− 2	0.1
352	1717	0.675	0.317	48	0.00660	0.320	0.677	1720	+ 3	0.2
365	1957	0.770	0.770	61	0.00860	0.406	0.718	1830	−127	6.5
416	2145	0.844	0.733	112	0.00646	0.745	0.848	2155	+ 10	0.5
445	2357	0.926	1.097	141	0.00777	0.940	0.897	2280	− 77	3.3
478	2547	1.000		174		1.160	0.936	2380	−167	6.5

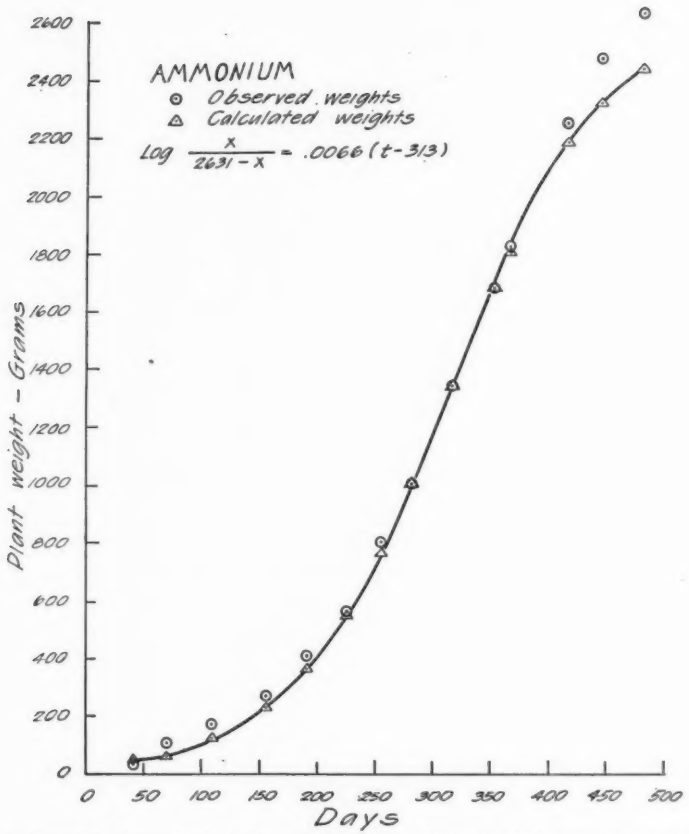

FIG. 1. Growth of pineapple plants in complete nutrient solution in which ammonium salts furnished the nitrogen.

plants were about 400 days old and influenced the values of the last 3 readings. The weight of fruits when about 16 days old was about 15 grams, of 45 days about 75 grams and of 78 days about 300 grams.

The data were reduced to fit the monomolecular reaction formula, following Robertson's procedure, where

$$\frac{dx}{dt} = k(A - x) \cdot \tag{1}$$

Equation (1) is modified by the fact that the velocity-constant K is multiplied by the mass (concentration) of a catalyzer which is proportional to the mass of the products of the reaction, as follows:

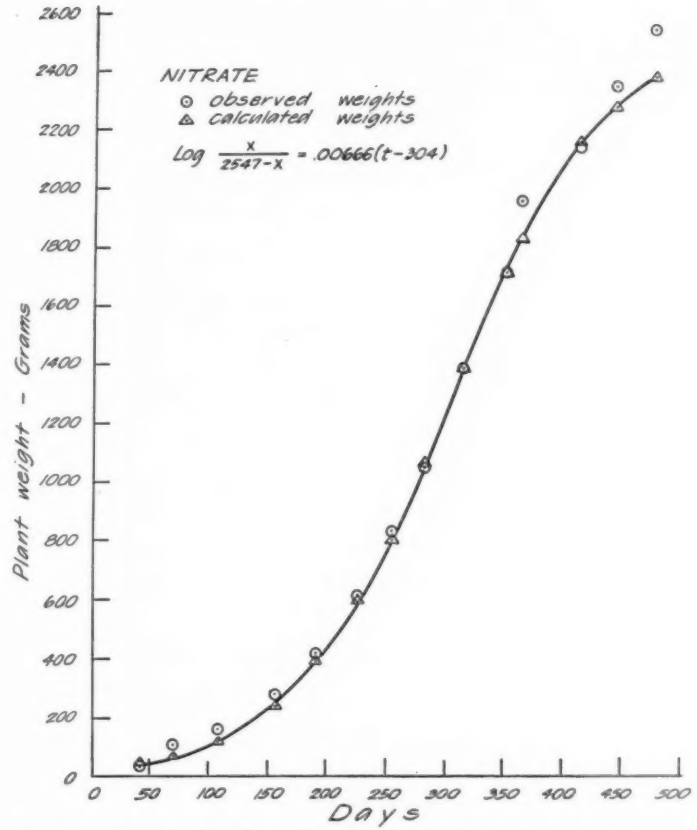

FIG. 2. Growth of pineapple plants in complete nutrient solution in which nitrate salts furnished the nitrogen.

$$\frac{dx}{dt} = k_1 k(k - k) \cdot \tag{2}$$

which, when integrated, yields the relation:

$$\log \frac{x}{A - x} = K(t - t_1) \tag{3}$$

where $K = k_1 A$ and t_1 is the time at which the reaction is half completed, that is, when $x = (\frac{1}{2})A$. The values of $K(t - t_1)$ were obtained from Robertson's (8) table LX on page 329.

RESULTS

The results in tables 1 and 2 and Figures 1 and 2 show that percentage deviations between observed and calculated values are comparatively smaller during

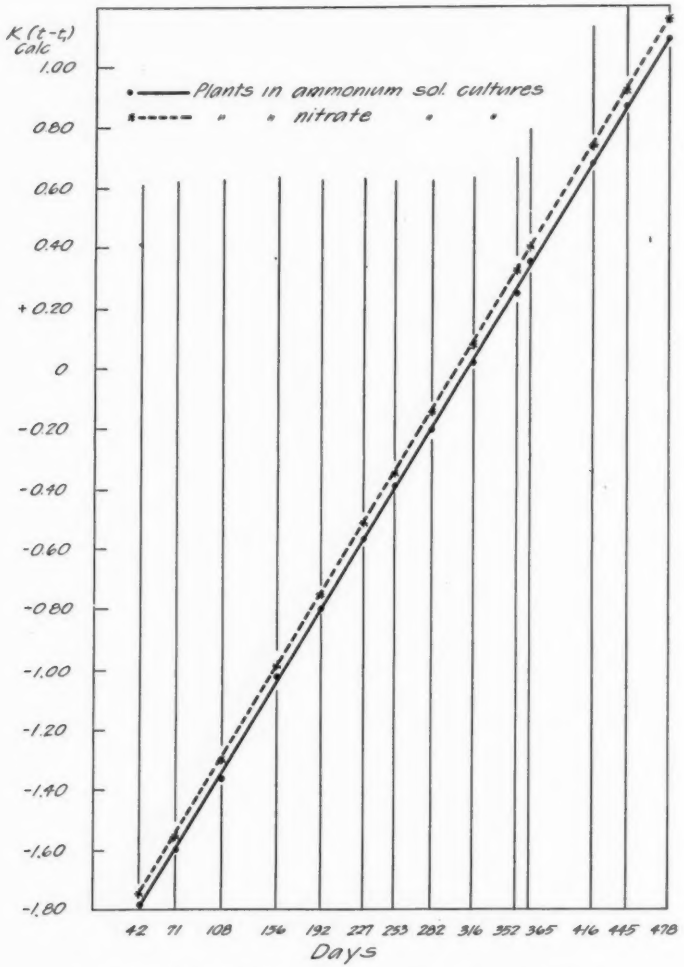

FIG. 3. Values of $K(t-t_1)$ from the equations for the growth curves.

the later than the earlier stages of growth. For the first 3 months the percentage deviations are very high for various reasons. During the first period, that is, from the beginning of the experiment to the 42nd day, the plants did not begin

growth until they had produced roots, which required the first 15 to 20 days of the period. During the second and third periods, terminated by the 71st and 108th days respectively, the rate of growth was comparatively greater than during the first period because of the complete recovery and establishment of the plants during these periods. Observed weight values of plants obtained during the first three periods of these experiments have always shown a greater divergence from calculated weight values than those of later periods. Excepting the first 3 periods, agreement between observed and calculated values of X are very satisfactory.

In Figure 3 the values of $K(t-t_1)$ of tables 1 and 2 have been plotted against time (t) to check the accuracy of the calculated values of X.

The values of either the observed or calculated weights of the ammonium and nitrate series of plants were, with minor exceptions, of the same magnitude indicating that the effects of either ammonium or nitrate on plant growth at pH 5.0 ± 0.4 were about the same.

DISCUSSION

The findings support the theory of Robertson and other investigators that the physicochemical reactions involved in growth are comparable and possibly similar to those in autocatalytic monomolecular reactions. The mathematical relations which express the conditions of an autocatalytic monomolecular reaction have been applied to the growth of pineapple plants with fairly satisfactory results.

However, with plants involving more than a single growth phase, that is, where fruit growth or that of other vegetative organs is simultaneous, the mathematical formula mentioned above is inadequate. Under such conditions each individual organ must be treated independently as each one represents an autocatalytic process clearly distinguishable from the growth-curve of the plant as a whole. Robertson (8) and Reed (6) have studied the various factors which determine the sequence of growth-cycles and explain quite satisfactorily the asymmetrical type of growth-curve obtained under such condition.

The relatively high divergence values during the last two readings may be attributed to an early termination of the experiment which was about 100 days before the stage of fruit ripening and during which growth of vegetative organs are at a standstill.

SUMMARY

The growth of pineapple plants in water culture with either ammonium or nitrate as the source of nitrogen in a complete nutrient solution follows the curve of an autocatalytic monomolecular reaction. The divergence between observed and calculated weight values of the first 3 periods are great on account of the readjustment of the plants to their environment.

The weight values of the plants of the ammonium series were approximately of the same magnitude as those of the nitrate series, indicating that both forms of nitrogen were equally effective in promoting plant growth when the pH was maintained at pH 5.0 ± 0.4.

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CELL NUMBER AND ORGANIZATION

By
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In an earlier paper (Berrill, '35) it was shown that in developing ascidian buds cell multiplication continues until a certain number of cells have been formed, and only then when cell division has virtually ended does histo-differentiation become apparent. A gill-slit first appears in the pharyngeal wall as a rosette of six or eight cells surrounding a small perforation of the wall. Only two or three further divisions of these cells occur, by which time they have become fully differentiated ciliated stigma cells. Whatever the total number of cells in the pharyngeal wall, the stigmata first appear, as rosettes, only when cell multiplication is ending. The number of independent stigmata or rosettes that can be formed is clearly limited by the total number of cells present in the pharyngeal wall. In the case of certain buds where there is no nutritional limitation cell, multiplication continues until there are sufficient for the formation of the full complement of stigmata characteristic of the species. In others where nutritional limitation exists, the number of stigmata and rows of stigmata that can be formed will be less than the specific numbers.

In the same paper it was shown that in the case of eggs there is a dual development. On the one hand there is an early determination of certain parts, commencing before fertilization, that culminates in the precocious formation of a tadpole organization when but one-fifth of the total course of cleavage (in time) has passed. The tissues concerned with tadpole structure terminate cell division at a very early stage, so that they consist of comparatively small numbers of large cells. On the other hand, the remaining parts of the egg, which are much the greater part, continue to divide until numerous small cells are formed that have a certain critical size characteristic of the species. As in bud development, no histo-differentiation occurs in this case until cleavage has virtually ended. The number of cells formed consequently varies according to the size of the egg and the amount of yolk it contains.

The purpose of this paper is to relate the number of cells finally formed by the cleavage of the egg, and to trace the course of development of the gill-slits, as an example of organization, in the various cases.

MATERIAL

The species studied were *Ciona intestinalis*, *Ascidiella aspersa*, *Styelopsis grossularia*, *Distomus variolosus*, *Stolonica socialis*, *Perophora listeri*, *Ecteinascidia conklini*, *Ecteinascidia turbinatum*, *Clavelina lepadiformis*, and *Clavelina picta*. The material was collected or reared at the Marine Laboratory at Plymouth and at the Bermuda Biological Station.

FINAL CELL NUMBERS

The genus with the most primitive egg is Ciona. The rest form three groups. The styelids, Styelopsis, Distomus and Stolonica all have eggs of the same type but of different sizes. Perophora and the two species of Ecteinascidia also have eggs that differ only in size, the yolk content being the same, though greater than that of Ciona or the Styelids. The two species of Clavelina have eggs that are somewhat yolkier again, but are themselves two sizes of the same type. Since the perophorids and clavelinids have both yolkier eggs than the styelids and Ciona, more cells should be produced by the end of cleavage as compared with eggs of the same size. The actual conditions are shown in Table I.

TABLE 1

A COMPARISON OF EGG SIZE, FINAL NUMBER OF CELLS FORMED, AND DURATION OF DEVELOPMENT

Species	Diameter of egg in mm.	Volume of egg in cu. mm.	Approximate number of cells formed	Cell number proportionate to volume	Duration of cleavage at 16°C. hours
Ciona intestinalis	0.16	0.0026	3,400	3,400	190
Styelopsis grossularia	0.48	0.0537	41,000	91,800	340
Distomus variolosus	0.59	0.1024	69,000	173,000	440
Stolonica socialis	0.72	0.1865	115,000	319,000	470
Perophora listeri	0.24	0.0069	14,500	10,370	190
Ecteinascidia conklini	0.58	0.0975	87,000	166,600	360
Ecteinascidia turbinatum	0.72	0.1865	173,000	319,600	460
Clavelina lepadiformis	0.26	0.0092	17,500	15,640	280
Clavelina picta	0.48	0.0537	64,000	91,800	390

It is evident that the total number of cells formed during cleavage increases with the volume of the egg and that the number of cells is further increased when the proportion of yolk in the egg is greater, as in the perophorids and clavelinids as compared with the styelids. At the same time it is equally clear that, with two exceptions, the total number of cells is less than half the expected number. This difference however practically disappears if the theoretical number is divided by the number of times the duration of cleavage of the species concerned is greater than that of the Ciona egg.

An explanation is that a certain amount of substance of an egg is consumed in metabolism per unit of time during the total period of cleavage. The longer the period of cleavage the greater the percentage of substance consumed.

DEVELOPMENT OF STIGMATA

The course of development of gill-slits or stigmata varies greatly among ascidians (cf. Willey, 1892; Damas, 1900; Julin, '04; Fechner, '07; Huntsman, '14; and Garstang, '28). Previous discussion has however centered chiefly around the recapitulatory significance of such development and little attention has been paid to its relation to body size or cell number.

In the case of zooids developing from small eggs, such as those of Ciona,

Ascidia, and Styela, two protostigmata at first appear and function on each side of the pharynx. Only with post-functional growth does the number increase. The protostigmata on each side, by derivation or by independent perforation, increase in number until six are formed, when each divides and subdivides with further growth until six rows of definitive stigmata are formed. In those forms where two peribranchial siphons are present in the newly functional ascidiozooid, it is at this stage that they fuse to form the single atrial siphon characteristic of all adults.

The first pair of perforations appear in each branchial wall when it consists of about 80 or 90 cells, each perforation being the centre of a rosette of about 20 cells. By the time the cells of a rosette have become fully differentiated as stigma cells, they number about 160. The manner in which the number of protostigmata is raised to six is not the immediate purpose of this paper to describe, but during this process each protostigma gradually increases in length until it consists of about 320 cells, after which it constricts into two. Growth and subdivision of a stigma as a whole thus resembles remarkably growth and subdivision of a cell.

TABLE 2

CELL NUMBERS OF STIGMATA AND ZOOID

Species	Stage	Number of cells per stigma	Total number of cells of all stigmata	Total cell-number of ascidiozooid
Ciona intestinalis	C	160	680	3,650
	G	180	3,600	15,300
	H	180	7,400	30,300
	I	180	17,280	49,500
Ascidia aspersa	=C	160	640	3,400
	K	160	7,200	31,500

Table II and Figure 1 illustrate the cell numbers associated with the various stages of stigmata multiplication and peribranchial fusion in Ciona and Ascidiella oozooids. It is evident that a differentiated stigma has to double its cell number in order to subdivide, and that fusion of the peribranchial siphons to form the single atrial siphon is associated with a total number of stigma cells of about 7,000, and a total number of cells for the organism of about 30,000. The first division of the protostigmata occurs when the total cell number is about 10,000.

In the case of species with larger or yolkier eggs, histodifferentiation of branchial structure is still postponed until but two or three divisions remain before cleavage ends. Consequently the cell number associated with protostigmata stages or with fusion of peribranchial siphons may be attained and passed long before the onset of histodifferentiation.

This is definitely the case for Distomus, Stolonica, *Ecteinascidia conklini*, *Ecteinascidia turbinatum* and *Clavelina picta*. Histodifferentiation appears when the total cell numbers of developing ascidiozooids are already characteristic of rows of definitive stigmata and a median atrial siphon. Consequently the stigmata appear from the beginning in these forms as rows of independent perforations, the number of rows varying with the total number of cells constituting the pharyngeal wall. In Distomus four rows first appear, in Stolonica eight;

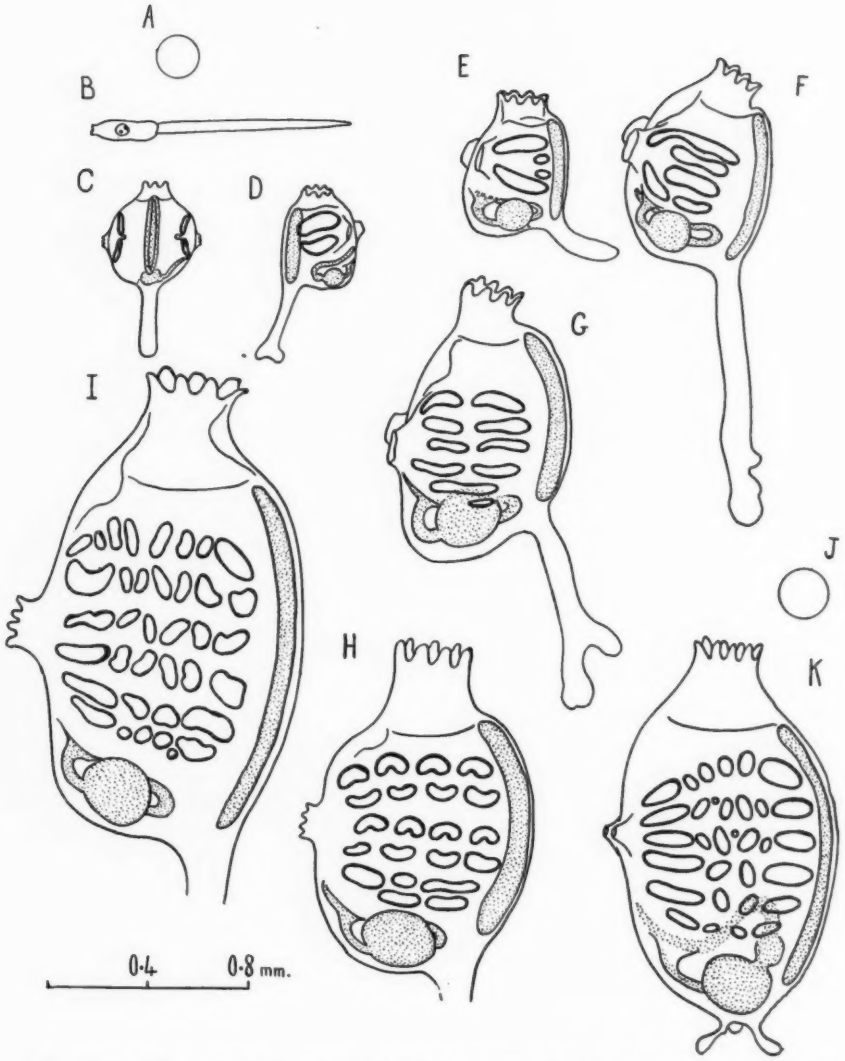

FIG. 1. Development of stigmata and atrial siphon in Ciona intestinalis (*A-I*) and Ascidiella aspersa (*J-K*). *A* and *J* indicate the size of the eggs relative to that of the zooids developing from them. *C* and *D*, ventral and lateral aspects of the oozooid of Ciona when newly functional. *E-I*, progressive formation and subdivision of protostigmata to form rows of definitive stigmata. In *H* and *I* the lateral peribrachial siphons have fused to form the median atrial siphon. In *K*, Ascidiella the fusion is almost complete.

in *Ecteinascidia conklini* six rows, in *E. turbinatum* twelve rows appear. In the case of Styelopsis, Perophora and *Clavelina lepadiformis*, however, the total cell number attained at the moment of onset of histodifferentiation is at a critical value.

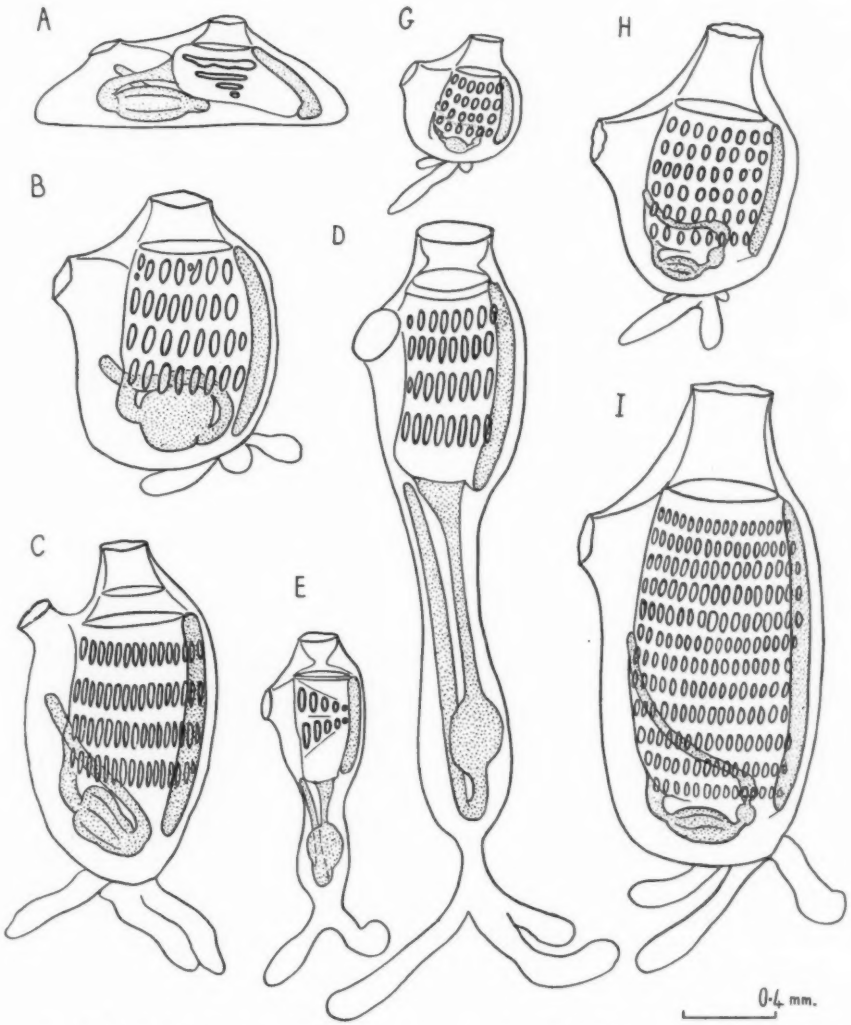

FIG. 2. Newly functional oozooids drawn to the same scale as that of Ciona shown in Figure 1, of various species developing from larger eggs than those of Ciona and Ascidiella, to show the relation of stigma number to material available. *A*, Styelopsis grossularia; *B*, Distomus variolosus; *C*, Stolonica socialis; *D*, Clavelina picta; *E*, Clavelina lepadiformis; *G*, Perophora listeri; *H*, Ecteinascidia conklini; *I*, Ecteinascidia turbinatum.

In Styelopsis at this stage the total number of cells is such that there are available sufficient only for the formation of the full number of protostigmata (six) on each side, and their subdivision into rows of definitive stigmata depends upon a certain amount of growth resultant upon feeding.

In Perophora and in *Clavelina lepadiformis* the cell number attained when histodifferentiation begins is typical of protostigmata and paired peribranchial siphons. Consequently both protostigmata and lateral siphons appear. But during the final two or three cleavages the cell number is raised to a value characteristic of rows of definitive stigmata and of a fused median atrial siphon. This occurs while histodifferentiation is in progress and before functional differentiation is attained. Therefore the transition from the protostigmata to the definitive stigmata stage is an embryonic rather than a post-functional phenomenon.

The course of development of such structures as the gill-slits of ascidians is accordingly a function of the number of cells attained by the developing embryo during its closing phase of cleavage. If the presence of protostigmata in ascidian development has significance relative to the theory of recapitulation, the fact that they are definitely associated only with organisms having less than a certain number of cells, or below a critical size, must be taken into full consideration.

SUMMARY

The total number of cells formed as the result of cleavage varies with the egg volume and yolk content, though not directly. When allowance is made for the duration of cleavage the relationship becomes proportional. An explanation is that a certain quantity of egg substance is consumed, per unit of time, in metabolism.

Stigma cells, and cells of all permanent tissues, vary in number more or less directly with the egg mass. Stigmata perforation occurs when cleavage is within about three divisions of the end of its course, marked histodifferentiation occurring only after the last division.

Protostigmata and lateral peribranchial siphons are to be correlated with cell numbers below a certain critical value. When the total cell number exceeds this value, rows of definitive stigmata and a median peribranchial or atrial siphon develop directly. When the critical cell is attained during the last two cleavages of an egg, the transition from the protostigmatal to the definitive stigmatal stage is an embryonic phenomenon.

The interpretation of developmental stages in terms of the theory of recapitulation must take full consideration of the relationship existing between absolute size or cell number and the organization of the embryo, larva, or young individual.

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THE COURSE OF POPULATION GROWTH AND THE SIZE OF SEEDING

ILLUSTRATED WITH YEAST

By

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The growth of a population depends on the favorableness of the environment, the number of individuals present in it and their ability to use the materials present. Unless the environment be maintained effectively constant it becomes unfavorable. Yeasts are appropriate experimental organisms as the environmental changes may be controlled and studied. However, it is technically difficult always to seed into a given amount of culture medium the same number of physiologically similar cells. In successive experiments under as nearly identical conditions as possible the growth is found to be of the same general nature but each population shows some slight variation.

A true picture of the growth process cannot be obtained by directly averaging the observations obtained from different experiments nor is it feasible to fit a general equation to each set of observations and to average the resulting constants to obtain a general curve of yeast population growth. Under certain conditions the method to be developed in this paper makes it possible to extend the analysis of the growth function of yeast with especial reference to the effect of seeding different amounts of yeast into the same environment.

I

The rate of growth of a population of yeast, when the cells are able to adapt themselves quickly to such changes as may occur in the medium during the course of an experiment, depends upon the number of individuals present and the condition of the culture medium, or the concentrations of foodstuffs and of excretion products. These facts may be expressed analytically by

$$\frac{dN}{dt} = N \cdot F(p_1, \cdots, p_n), \quad \text{or} \quad \frac{1}{N}\frac{dN}{dt} = F(p_1, \cdots, p_n),$$

where N stands for the number, mass, volume or other measure of the population, the p's represent the concentrations of materials in the medium, t is the time, and $F(p_1, \cdots, p_n)$ is the value of the relative growth rate, which is dependent on the p's.

Changes may occur in the environment, such as evaporation, oxidation, etc., which are not functions of the yeast growth. These changes are apparently not of sufficient importance during most of the growth in the present experiments to justify increasing the complexity of the analysis to include them, but some of the variation to be described later may be due to them.

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The p's then are functions of the amount of growth which has occurred: $p_i = p_i(N - N_0)$, where N_0 is the size of the seeding from which the population was started. If the size of the seeding is so small that the changes in the medium which would result from the production of an equal number of cells could be neglected, no appreciable error is made by using N in place of $N - N_0$.

The relative growth rate depends on the values of the p's and they in turn are determined by the amount of growth that has taken place; therefore the relative growth rate is a function of the amount of growth (Lotka, '25). The differential equation may be changed to

$$dN/Ndt = f(N - N_0),$$

or, if N_0 is sufficiently small, to

$$dN/Ndt = f(N).$$

The amount of growth $(N - N_0)$ may be designated by n for convenience and then

$$f(N - N_0) = f(n).$$

II

The application of the equations above to experimental conditions requires that the different initial conditions be identical except for the differences in the amounts of seed yeast. Such experiments have been made, and the data may be used to test and verify the above hypotheses.

If the relative growth rate (dN/Ndt) is calculated for a population from time to time and plotted against n, i.e., the amount of population produced in the medium, the resulting graph gives a picture of $f(n)$ as a function of n, or the change of the relative rate of growth as the population increases by the addition of new individuals. On the basis of the above assumptions the data from the several populations should all produce the same curve within the limits of error of the methods used.

The verification of the hypotheses stated permits certain conclusions concerning the nature of the metabolic processes accompanying the proliferation of the yeast. Food is consumed, not only for the production of new cells, but also for the maintenance of the cells already present. Growing populations started with large seedings deplete the medium more rapidly, in proportion to the number of new individuals produced, than those started with small seedings. When a considerable amount of food is utilized for the maintenance of life, the plots of the relative growth rate, $f(n)$, against n will give different curves for the populations from different sized seedings. A similar analysis of the production or depletion of an injurious waste substance will yield a like result: e.g., the removal of a waste substance by a process not directly connected with growth, such as the loss of alcohol by evaporation, is not consistent with the hypotheses, unless the removal is so rapid that the remaining amount is negligible.

A certain caution is to be observed in the practical application of the con-

clusions. It is conceivable that in some experiments effects of the general nature of consumption of food for maintenance, which would tend to render the medium less favorable, and removal of deleterious products, which would tend to improve the medium, might approximately balance each other, irrespective of the proliferation of the yeasts. Until definite knowledge of the detailed processes taking place becomes available, we may conclude that the *net result* of such processes is negligible.

When the above assumptions and conclusions are verified, a method becomes possible for "averaging" growth curves of populations started from different sized seedings. It is well known that averaging from actual counts (or weights, etc.) of the different populations involves difficulties, since the courses of growth of different populations cannot be considered to be identically the same. When the $f(n)$ is the same function for all the populations, the varying observed values of dN/Ndt corresponding to a given value of n can be considered as approximations to the true value of $f(n)$, and therefore can be averaged to give a result which may be taken as the best estimate of the true value.

Finally, when the function $f(n)$ has thus been experimentally obtained, its nature may be studied further and expressions for it and for the curve of growth may be derived.

III

In the experiments under consideration, eight populations were grown in portions of the same medium, under as nearly identical conditions as possible, differing from each other only in the amounts of seeding. The seed yeasts came from the same population and the complications of any lag period were avoided.

THE GROWTH OF EIGHT YEAST POPULATIONS

Number of cells per 1/250th cu. mm.

Time, hrs.	I	II	III	IV	V	VI	VII	VIII
0*	.06	.17	.33	.50	.66	1.32	2.64	5.28
5	.19	.23	1.0	1.0	1.7	3.4	5.2	10.0
17	1.3	1.4	4.0	6.6	10.0	13.0	20.6	24.6
29	3.5	7.8	13.5	15.2	24.1	25.3	41.9	50.3
41	12.6	24.9	37.4	41.1	46.6	42.1	55.3	55.2
53	28.6	36.4	45.6	43.9	65.8	65.0	67.8	57.1
65	41.6	52.8	50.4	57.0	65.9	78.7	79.6	82.8
77	61.6	61.6	70.0	65.0	65.8	73.6	84.8	88.3
89	68.3	70.2	78.3	86.1	85.9	75.0	82.6	75.6
102	77.2	74.8	75.3	78.7	83.2	82.2	74.9	83.1
114	83.6	89.2	90.9	89.9	103.0	97.3	88.7	86.8
125	91.9	95.0	99.8	105.1	102.6	104.4	101.9	90.7
137	98.3	93.5	89.4	92.9	89.6	89.4	96.3	83.9
162	96.7	101.7	81.0	89.5	93.0	85.2	89.7	84.7
186	90.5	83.0	86.1	100.5	83.7	93.3	82.6	99.3
213	93.1	88.5	87.7	81.5	87.7	91.8	93.8	93.1
N_0 corrected	.048	.097	.360	.401	.872	1.34	2.79	4.87

* The values for the seedings, i.e., for time = 0, are estimates from the volumes of inoculations, rather than direct counts.

The methods and necessary precautions have been published elsewhere (Richards, '32) and the counts of the numbers of cells present in the populations were made at intervals as shown in the table.

The further development of the theory is simplest if Napierian logarithms to the base e are used, but the calculations are made more easily with common logarithms to the base 10. In order to avoid the confusion of frequent shifts from one system to the other the logarithms to the base 10 will be used as far as practicable.

From the theory of logarithms,

$$f(n) = \frac{1}{N}\frac{dN}{dt} = \frac{1}{M}\frac{d}{dt}\log N,$$

M being the conversion factor of the two systems of logarithms ≈ 0.43429. Consequently we may obtain values of $f(n)$ for any population by taking the logarithms of the values of N, finding the differences between successive logarithms, and dividing each difference by the product of M and the length of the corresponding time interval. (It was found convenient in this case to adopt a temporary time unit of 12 hours and to postpone division by M, but the necessary adjustments were made later to give proper results in time units of one hour.)

Strictly speaking, we have now determined the mean growth rates for the several time intervals. By the theorem of the mean, however, each one is the actual rate at some instant within the corresponding interval. To approximate the value of N at that instant we take the geometric mean of the values of N at the beginning and end of the interval; this is easily done with the aid of the values of log N already obtained. Subtraction of the seeding value N_0 from each of these intermediate values of N yeast cells gives the values of n corresponding to the values of the relative growth rate, and we can now plot $f(n)$ as a function of n.

The points of the eight curves are graphed in Figure 1 for n between 0 and about 95. From this figure, or from separate plots of each of the eight curves, it is evident that the curves all have the same general shape. From comparatively high initial values the function decreases to zero as n increases, and the curves are concave upward. There is wide variation in the values of $f(n)$ for small values of n, and in two cases (populations II and IV) there is an initial rise before the decrease. For greater values of n the curves seem to be in good agreement until $n = 75$. Since the yeast growth rate has been shown to be constant under such uniform conditions as existed during early growth (Richards, '34) it seems reasonable to assume that the variation in the values when n is small is due to the inherent difficulties of accurately sampling and counting small populations. In addition, the seeding values given are not all direct counts. Allowing for part of this early variation as errors of estimate, then the data of the various populations (at least as far as $n = 75$) give the same function of n.

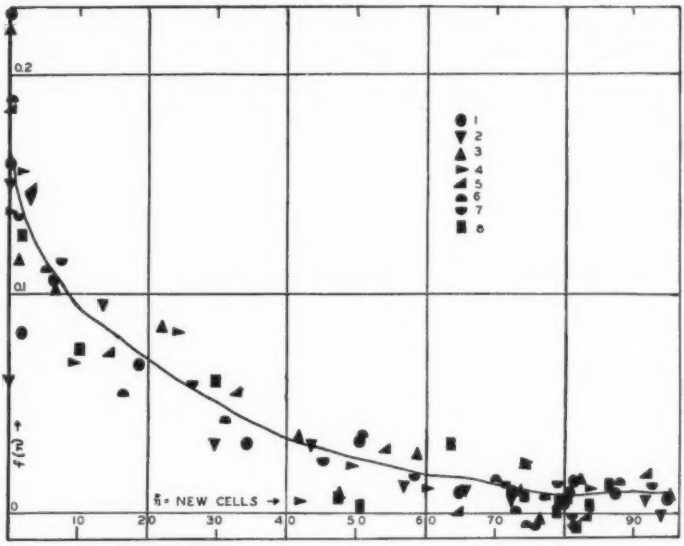

FIG. 1. The relative growth rate, $f(n)$, as a function of the number of new cells, n, produced in the medium for eight different initial populations.

The nature of the function may be studied with the aid of a rapid method for obtaining an average curve. The values of $f(n)$ corresponding to $n = 1, 2, 3, 4, 5, 10$ and thence by 10's to 80 were computed for each population by interpolation. The eight resulting values of $f(n)$ for each n were averaged and plotted to form the curve of Figure 1. The curve is concave upward, sloping sharply at first and then more gradually as n increases.

IV

It is useful to represent the $f(n)$ by a formula. Since we make no hypotheses concerning the nature of the function our only requirements are that the expression represent the data accurately and that it be easily handled in the subsequent uses to which we wish to put it.

The logistic curve of Verhulst and of Pearl and Reed, sometimes called the autocatalytic curve, has been used to represent the growth of yeast populations (Kavanagh and Richards, '34). The differential equation of their curve is usually stated as

$$\frac{dN}{dt} = kN(a - N); \quad a, k \text{ constants}$$

or

$$\frac{1}{N}\frac{dN}{dt} = ka - kN.$$

If the effects of different sizes of seeding are to be considered it should be written

$$\frac{1}{N}\frac{dN}{dt} = ka - kn.$$

The function $f(n)$ is a linear function of n and its graph will be a straight line. However, our results show that for the yeast and the culture medium used in these experiments the function $f(n)$ is curvilinear, Figure 1, and the logistic does not accurately represent the course of growth of this yeast, although it may furnish an approximation sufficiently accurate for many purposes.

The curve of averaged values suggests an exponential function:

$$f(n) = 10^{-(an+b)} \tag{1}$$

The values of the constants a and b may conveniently be determined from the curve $nf(n)$ instead of from $f(n)$ itself because the importance of the values of $f(n)$ in determining the course of growth depends not only on their size but also on the size of the population to which they correspond. By fitting a curve to $nf(n)$ the values of $f(n)$ are weighted by amounts which make them of approximately equal importance. Furthermore, the study of the graph of $nf(n)$ against n enables us to obtain the first approximations to a and b very quickly, as the following theory shows.

Let us determine the value of n for which $n \cdot 10^{-(an+b)}$ is a maximum. Set for simplicity

$$G = n \cdot 10^{-(an+b)},$$

then

$$\frac{dG}{dn} = \left(1 - \frac{an}{M}\right)10^{-(an+b)}.$$

For the derivative to become zero, the factor in parentheses must also vanish. Consequently

$$1 - \frac{an_{\max}}{M} = 0$$

or

$$a = \frac{M}{n_{\max}}, \tag{2}$$

n_{max} denoting the value of n at the maximum point. Then

$$\begin{aligned} \log G_{\max} &= \log n_{\max} - an_{\max} - b \\ &= \log n_{\max} - M - b \end{aligned}$$

or,

$$b = \log n_{\max} - \log G_{\max} - M. \tag{3}$$

To approximate a and b we graph $nf(n)$ against n, estimate the values of n and $nf(n)$ at the maximum point and use them as n_{max} and G_{max} respectively in formulas (2) and (3). This procedure with our data gave $a=0.0161$ and $b=0.8066$.

Closer approximations were obtained by applying the conditions that the sum of the differences between the observed and the calculated values be zero, and that the theoretical and observed values coincide* for $n=3$. The first such approximation yielded $a=0.0156$ and $b=0.8362$, and the second gave $a=0.01560$ and $b=0.8368$.

To proceed to the integrated form of the curve (1) it is convenient to change from common (base 10) to Napierian (base e) logarithms:

$$\frac{1}{N}\frac{dN}{dt} = e^{-(cn+d)}.$$

By use of the conversion factor,

$$c = \frac{a}{M}, \quad \text{and} \quad d = \frac{b}{M}$$

and therefore the values of the constants become $c=0.03592$ and $d=1.9268$.

An additional approximation, using the integrated form of the curve, changed the values to $c=0.04013$ and $d=1.8416$ which were finally used.

Substituting for n its value, $N-N_0$, separating variables and integrating, we have

$$\frac{1}{N}\frac{dN}{dt} = e^{-(cN-cN_0+d)};$$

$$\frac{e^{cN}}{N}dN = e^{cN_0-d}dt;$$

$$\int_{N_0}^{N} \frac{e^{cN}}{N}\,dN = e^{cN_0-d}t. \tag{4}$$

The expression on the left cannot be integrated explicitly in finite terms of elementary functions of N. However, let $cN=-P$ and then

$$\int_{N_0}^{N} \frac{e^{cN}}{N}\,dN = \int_{-cN_0}^{-cN} \frac{e^{-P}}{P}\,dP = \int_{\infty}^{-cN} \frac{e^{-P}}{P}\,dP - \int_{\infty}^{-cN_0} \frac{e^{-P}}{P}\,dP \tag{5}$$

Following the customary mathematical notation, let us set

$$\int_{\infty}^{-x} \frac{e^{-P}}{P}\,dp = Ei(x).$$

* The latter condition was used because the weighting resulting from the use of $nf(n)$, discussed above, is less satisfactory when n is small; and our objective was a good description of the growth-rate during early growth. The final approximation, obtained later with integrated growth equation, eliminated the possibility of any considerable error resulting from this singling out of a special value.

Substitution in (5) and then in (4) gives

$$Ei(cN) - Ei(cN_0) = te^{cN_0-d}$$

$$Ei(cN) = te^{cN_0-d} + Ei(cN_0). \tag{6}$$

Tables of the function $Ei(x)$, which is known as the *exponential integral*, have been published, and it can be used as easily as the more common logarithmic and trigonometric functions. The principal tables suited for this work are those of Glaisher (1870), Jahnke und Emde ('23), and of the British Association ('31). The Glaisher table was found most convenient in the present work, although that of Jahnke und Emde (a condensation of Glaisher) would have been satisfactory. The British Association table has the disadvantage that it is necessary also to use a table of natural logarithms over the range in which we are interested. For the theory of the function the reader is referred to the descriptions accompanying these tables, or to Nielsen ('06), who gives an extensive list of references.

Students of growth-curve literature will notice that the curve does not approach an upper finite limit, as a theoretically perfect curve should. This difficulty is not of great importance in this case, since the formula is used only for the first growth cycle.

V

Since c and d are constants for this yeast under the conditions in which it was grown and since N_0 is constant for each population, it follows from (6) that for any given population the value of $Ei(cN)$ is a linear function of the time, and if it is plotted against t, as log N is plotted in a semi-logarithmic graph, the figure will be a straight line whose slope is e^{cN_0-d} and whose intercept on the vertical axis is $Ei(cN_0)$. In Figure 2 we have drawn the lines representing the theoretical courses of four of the eight populations, and have plotted the courses actually observed. Only four populations are shown in order to avoid overcrowding the graph. (Since the accuracy of the original values given for N_0 is open to question, as noted above, the "corrected values" given in the table were derived, and used in drawing the lines of the graph.)

The formula gives a good representation over the range in which it was fitted: that is, up to about the point where $n = 80$. This is approximately the level at which the sugar present in the medium is exhausted and where the first cycle ends. Beyond this point the general trend of growth up to 114 hours is shown by the formula, but it does not predict certain systematic fluctuations which occur. When a level of about 77 cells has not been surpassed by 102 hours (pops. I and II) the population continues to grow through the second cycle to its final maximum along the same curve. Among the populations started from the larger seedings there is a tendency to rise above the curve as the level, $N = 85$, is approached. The population then drops to a minimum at about the critical value of 77 cells, the minimum usually occurring at 102 hours, though in two cases it occurred earlier. The curves rise to maxima at 125 hours, with the exception of populations I and II whose maxima occur later: these maxima are

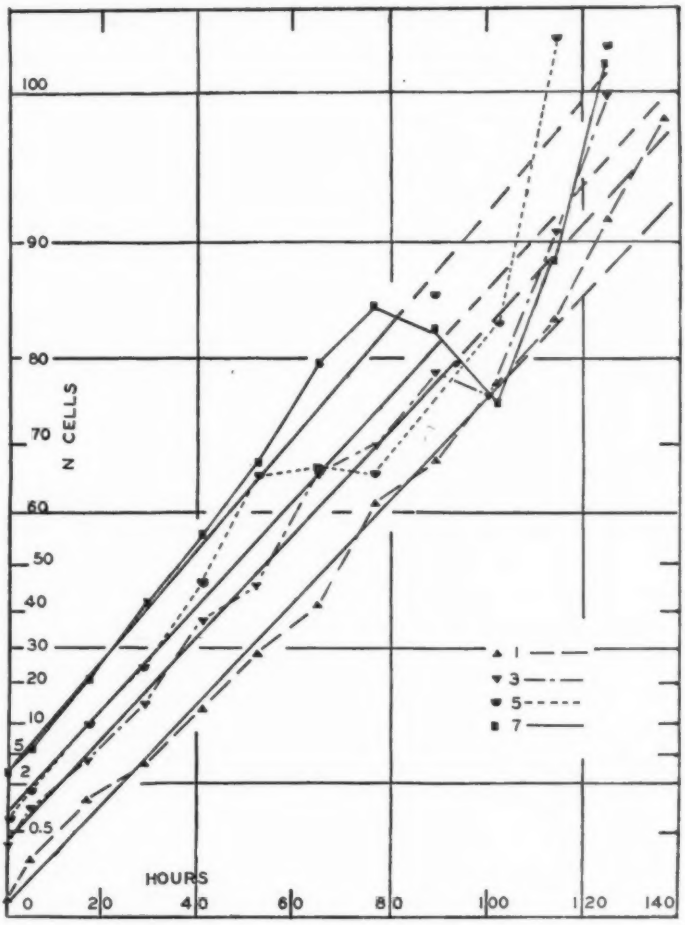

FIG. 2. The growth of four of the eight populations (*cf.* text).

usually greater than the values predicted by the formula for the corresponding times.

The fluctuations, beyond $n=80$, depend on N_0 as well as n and indicate something of the other factors, not expressible as functions of n alone, which affect the rate beyond this point. Some of the environmental factors which produce the second cycle of growth are known to be concerned with the material liberated into the medium from the disintegration of the killed cells and a reduction of the alcohol concentration in the medium.[2] With more information it will be possible to extend the analysis beyond the present limitation when $N=85$ cells.

SUMMARY

The relative growth rate of a population results from the condition of the culture medium; if this condition is determined completely by the amount of population increase previously taken place in the medium: then $\frac{1}{N}\frac{dN}{dt}=f(N-N_0)$, where N is the size of the population, N_0 the amount of seeding and t the time. The study of the growth of actual populations of an organism, started in exactly similar media with seedings of different size, furnishes a method for testing the validity of the hypothesis in a given experiment, because each population should yield the same values of $f(N-N_0)$ for all sizes of seedings.

If the assumptions are found to be verified experimentally, then the net results of actions tending to change the condition of the medium: but not directly connected with growth, such as consumption of food for maintenance as distinguished from consumption for reproduction, or the removal of wastes by evaporation, must be negligible.

The different curves may then be averaged by combining the separate results for $f(N-N_0)$, since they represent observations of the same function. (The direct population measurements cannot be so averaged, since the different growth curves do not have the same course.) From the results of such averaging the nature of the function $f(N-N_0)$ can be studied and approximate expressions for it and for the curve of growth can be derived.

The principles were applied to measurements of yeast growth previously reported by Richards. The combination of the results for the separate populations yields for $f(N-N_0)$ a curve which has the general nature of an exponential curve, rather than that of the logistic, or autocatalytic curve.

Thus $$\frac{1}{N}\frac{dN}{dt} = e^{-(cN-cN_0+d)},$$

and $c=0.04013$ and $d=1.8416$. The integration of the differential equation gives the form

$$Ei(cN) = te^{cN_0-d} + Ei(cN_0).$$

$Ei(x)$ is a function for which tables are available. This equation can be used to obtain a straight-line plot of the theoretical curves which facilitates comparison of theory and observation. In the present case the results of the treatment indicate that the assumptions are justified and that the formula is a reasonably good representation for the duration of the first cycle of growth.

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COMPARATIVE STUDIES ON THE GROWTH CAPACITY OF TISSUES FROM EMBRYONIC AND ADULT CHICKENS

By

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It is generally accepted that the tissues of adult organisms have a smaller capacity for growth than the tissues of embryonic organisms. This conception is based on the fact that as differentiation proceeds, the intensity of proliferation of the cells in an embryonic organism steadily decreases.

It is not clear whether this decrease in the intensity of proliferation is due to the cells themselves gradually losing this capacity by reason of the loss of certain inherent properties or whether environmental conditions appear within the organism as a whole which limit the growth of the cells.

This question could be decided by investigating the growth potentiality of cells derived from an adult organism but not influenced by the organism. The tissue culture method makes it possible to thus isolate tissues and measure their growth potentiality.

In our work, we endeavoured to examine growth potentiality with cell colonies isolated from the adult organism according to Carrel's method. We set out to determine the following: *Under identical conditions in vitro does a cell colony originating from an adult organism proliferate with the same intensity as one derived from an embryo?*

Carrel and Parker made studies of the relation of the growth rate of cells to the age of the organism from which they are taken. Both authors, however, worked only on tissues derived from embryos of different ages. Tissues of adult organisms have already successfully been cultivated (Moellendorff, Ephrussi and others) but no quantitative study of the growth of adult tissue in vitro has been made hitherto. Nor has a comparative study been made of the growth capacity of adult and embryonic tissues—as far as is known to us.

In our investigations we examined the growth capacities of tissues derived from 7 day old embryos, 19–21 day old embryos and of tissues of adult chickens (about one year old). Heart fragments of a uniform size were used for explantation. The tissues were cultivated in flasks according to the method of Carrel. Chick's plasma diluted with Thyrode in proportion 1:2 and a 30 per cent chick embryonic extract were used as media. The tissues of the three different aged organisms were cultivated either in separate flasks or altogether in the same flask. The cultures were projected every day and were drawn and measured with a planimeter in accordance with Ebeling's method. The resulting values were used in plotting the growth curves.

The results of our experiments are obvious. A comparison of the curve in the Figures 1 and 2 with Figures 3 and 4 shows that the growth rate of the tissues derived from older embryos is not below that of tissues from young embryos. Furthermore a comparison of the curves in Figure 1 to 4 with those in Figures 5 and 6 shows that fragments of adult heart have at least the same growth capacity as those of embryonic heart.

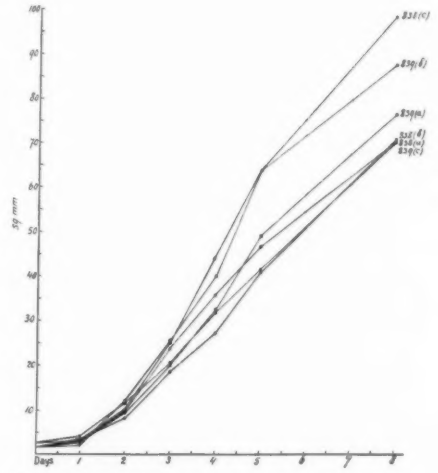

FIG. 1. The growth of heart fragments of 7 day chick embryos.

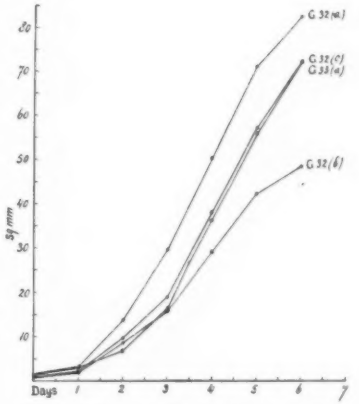

FIG. 2. The growth of heart fragments of 7 day chick embryos.

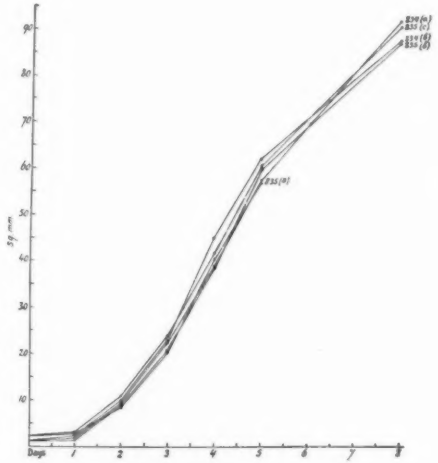

FIG. 3. The growth of heart fragments of 20 day chick embryos.

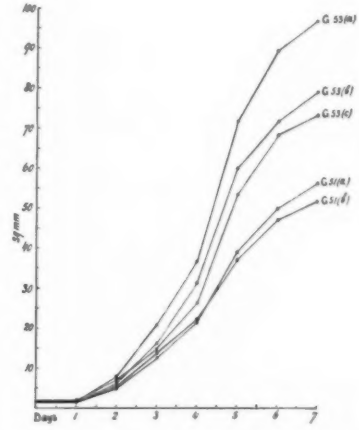

FIG. 4. The growth of heart fragments of 20 day chick embryos.

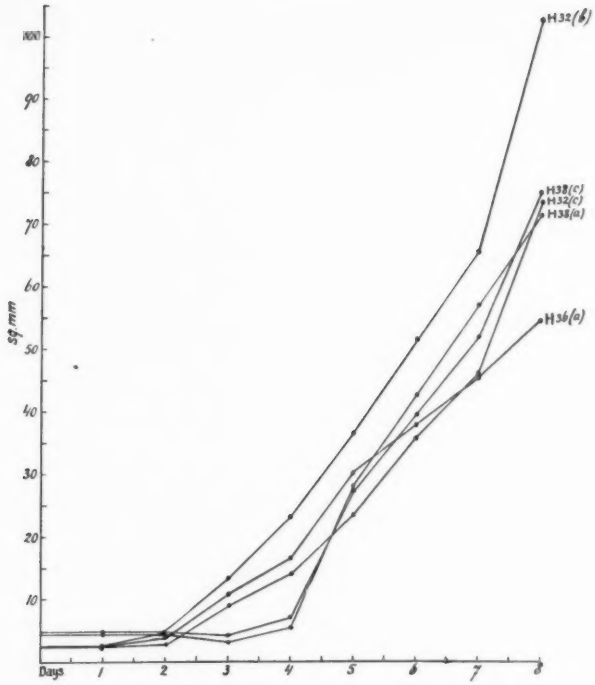

FIG. 5. The growth of heart fragments of 1 year old fowls.

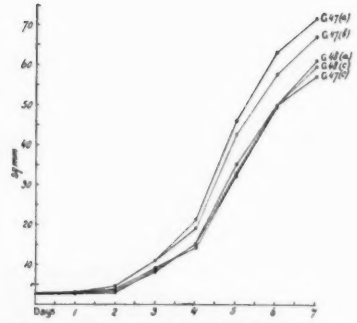

FIG. 6. The growth of heart fragments of 1 year old fowls.

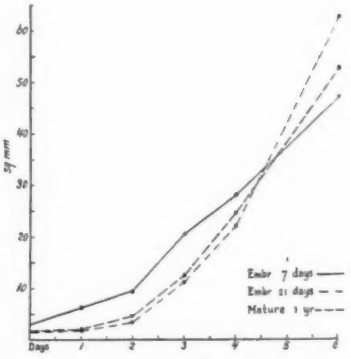

FIG. 7. The growth of heart fragments of 7 day embryos, 21 day embryos and adult fowls.

——— 7 day old embryo
– – – – – 21 day old embryo
-.-.-.-.-.- 1 year old fowl

TABLE 1
(To Figures 1, 2)

Culture No.	Size of culture in mm. after 0	24	48	72	96	120	144	168	192 hours
838 (a)	2.4	4.0	11.6	20.5	31.7	41.6	—	—	70.1
838 (b)	1.7	3.4	8.0	18.6	27.0	41.0	—	—	71.0
838 (c)	1.5	2.0	11.8	25.4	40.0	63.8	—	—	98.5
839 (a)	1.6	2.8	9.4	20.1	32.5	49.2	—	—	76.6
839 (b)	2.6	3.0	10.0	25.1	44.5	63.9	—	—	87.8
839 (c)	1.6	3.0	9.8	23.6	35.8	46.7	—	—	70.1
G 32 (a)	1.6	3.2	13.9	29.0	—	71	82.6	—	—
G 32 (b)	1.1	2.4	8.7	15.7	—	42.3	48.5	—	—
G 32 (c)	0.8	2.5	9.8	18.9	—	57.1	72.2	—	—
G 33 (a)	1.3	2.5	6.6	16.5	—	56.1	72.8	—	—

TABLE 2
(To Figures 3, 4)

Culture No.	Size of culture in mm. after 0	24	48	72	96	120	144	168	192 hours
834 (a)	1.7	2.0	9.4	22.5	38.6	57.2	—	—	91.5
834 (b)	1.9	2.2	9.9	23.0	45.0	62.0	—	—	87.3
835 (a)	2.2	2.7	8.5	20.0	40.2	57.0	—	—	—
835 (b)	1.7	1.8	8.9	20.8	38.5	59.7	—	—	86.6
835 (c)	2.2	2.9	11.0	24.4	41.6	60.8	—	—	90.5
G 51 (a)	1.2	—	4.9	12.6	21.4	39.0	49.9	56.1	
G 51 (b)	1.8	—	5.2	13.6	21.9	36.9	46.9	51.7	
G 53 (a)	1.5	—	7.9	21.2	36.4	71.7	89.2	97.0	
G 53 (b)	1.6	—	6.3	15.4	31.2	60.0	71.6	79.0	
G 53 (c)	2.1	—	7.1	14.8	26.3	53.5	68.2	73.2	

TABLE 3
(To Figures 5, 6)

Culture No.	Size of culture in mm. after 0	24	48	72	96	120	144	168	192 hours
H 32 (b)	2.6	2.6	4.2	14.7	25.1	39.3	58.5	65.5	102.6
H 38 (a)	4.5	4.5	4.5	3.3	5.6	28.2	—	57.0	71.5
H 32 (c)	2.6	2.6	3.0	9.0	14.2	23.5	35.7	46.1	73.4
H 36 (a)	2.6	2.6	4.0	11.0	16.8	30.3	38.1	45.5	54.7
H 38 (c)	5.0	5.0	5.0	4.6	12.1	27.2	—	51.9	75.1
H 36 (b)	2.5	2.5	4.8	13.5	23.3	36.6	51.8	65.6	—
G 47 (a)	2.6	2.6	4.5	11.1	21.3	46.1	62.9	71.6	
G 47 (b)	2.8	2.8	4.6	11.3	19.1	42.4	57.6	67.0	
G 47 (c)	3.1	3.1	3.4	8.3	15.2	35.1	49.7	57.1	
G 48 (a)	2.5	2.5	3.8	9.3	14.4	32.4	50.2	61.1	
G 48 (c)	3.2	3.2	—	8.8	15.4	32.8	49.6	59.6	

The curves which are given here were taken from a large amount of material, of which they give a true picture. Some of the curves show low, others quite high end-values, however, the majority of the curves are ranged between the two extremes.

The growth capacity of tissues, derived from 7 day old embryonic, 19 day old embryonic and adult chickens can be even better compared when they are all cultivated in one flask under absolutely identical conditions. The curves in Figure 7 give an idea of the rate of proliferation of these cultures. Table 5 gives the end-values of a number of such experiments.

TABLE 4
(To Figure 7)

	Size of culture in mm. after						
	0	24	48	72	96	120	144 hours
7 day embryo	3.0	6.5	9.9	20.8	28.0	—	47.2
21 day embryo	1.5	1.9	4.6	12.6	24.6	—	52.9
Adult fowl (1 year old)	1.7	1.8	3.5	11.3	21.9	—	62.7

TABLE 5
THE GROWTH OF HEART FRAGMENTS OF A 7 DAY EMBRYO, A 20 DAY EMBRYO AND A ONE YEAR OLD FOWL

	Size of culture after 96 hours in mm.	Size of culture after 144 hours in mm.
	Experiment No. 511	
7 day embryo	28.0	74.1
20 day embryo	31.9	64.4
Adult fowl (1 year old)	26.0	82.1
	Experiment No. 513	
7 day embryo	28.3	65.0
20 day embryo	38.3	74.2
Adult fowl (1 year old)	34.7	86.4
	Experiment No. 514	
7 day embryo	29.7	79.2
20 day embryo	30.0	66.7
Adult fowl (1 year old)	27.3	72.7

It is evident from the above experiments that colonies of cells derived from an adult organism may exhibit in vitro the same growth capacities as those of embryos.

In examining the growth cruves of cultures grown in separate flasks one is struck by the fact that in spite of the approximately identical *end-values* for growth of the explanted tissues from differently aged embryos and adults the *course* of the curves of these cultures definitely varied. At about six hours after explanting the heart fragment of a 5 to 7 day old embryo threadlike pseudopodia appear which soon develop into an area of growing cells. After about 24 hours the growth curve of young embryonic heart cultures rises sharply and follows the usual S-shaped course. The development of tissue fragments from 20 day embryo proceeds in an almost identical fashion, the only difference being the later appearance of the first signs of growth as compared with tissues of young embryos. The first new cells usually appear not in 6 hours but in 10 to 12 hours

after explantation, however, at the very moment of their appearance proliferation sets in immediately thus making it difficult to distinguish the growth curves of young and old embryonic tissues.* However the course of the growth curves of colonies of cells derived from adult heart differs markedly from those of embryonic heart. The first cells generally appear only after 28 to 35 hours and occasionally only after 70 to 120 hours. The curve from adult tissue is perfectly level for a time, and generally only after 48 hours does it follow the course of the curve of an embryonic tissue culture.

It is seen from the above that the *growth capacity* of cells derived from an adult organism is the same as that of embryonic cells; but the *course of growth* differs. *The cells from adult organisms must pass through a longer latent period in vitro before proliferation sets in.* The length of this period may vary.

We intend to deal in our next paper with the problem of the latent period in the growth of the tissues of the adult and embryonic organisms in vitro as well as that of latency variations and latency control.

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* The fact that the onset of growth in cultures from older embryos is later than in cultures of younger embryos was demonstrated by Cohn in 1925.

TEMPERATURE AND THE CLEAVAGE RATE OF *ARBACIA* AND *CHAETOPTERUS*

By

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The following report contains a description of the effect of temperature on the rate of cleavage of the eggs of the sea urchin, *Arbacia punctulata*, and the annelid, *Chaetopterus pergamentaceus*. The temperatures employed were not low enough to reveal the point at which cleavage is completely inhibited in *Arbacia* (specific zero; physiological zero; biological zero of Bêlehradek, 1935) though they were for the egg of *Chaetopterus*. In most respects the data are not greatly different from those accumulated by other workers for various forms, both vertebrate and invertebrate (see Atlas 1935 for the Amphibia; Gray 1928, and Worley 1933 for the fish; and Tyler 1936 a and b for various invertebrates).[1] For the most part these investigators have been concerned with the effects of temperature on the rates of various phases of the mitotic division, the rate of oxygen consumption, carbon dioxide production, and in one instance the respiratory quotient, and with the determination of the temperature coefficients for various developmental processes. The data presented here are interesting in that the equipment available permitted test at eight different temperatures to be run with the eggs of each female. A comparison of the results obtained on *Arbacia* with those obtained on *Chaetopterus* has led to conclusions concerning the rôle played by temperature in the distribution of animal species and, when supplemented by as yet unpublished data obtained by Hermes on the Amphibia, indicates a correlation between the breeding seasons of species i.e. environmental temperature, and the behavior of their eggs. Such a correlation has already been suggested by Lillie and Knowlton (1897) and by Hörstadius (1925).

The modification of developmental rate and pattern through the variation of the temperature of the environment, other conditions remaining as nearly constant as possible, has yielded valuable information of both an analytical and a descriptive nature. The first systematic study, made by Lillie and Knowlton on amphibians, demonstrated clearly that two more or less distinct effects are recognizable, one of which appears in the alteration of structure (i.e. form; more evident at subminimal and supramaximal temperatures), and the other of which is interpreted in terms of rate and is detectable over the entire developmental range. While the grosser aspects of development are normal over the interval between the minimum and the maximum[2] it is known from such investigations as those of Harnly and Harnly (1936) that phenotypic expression of genetic characters may be modified. Similarly, genetic and cytological in-

[1] See other references here.

[2] It should be borne in mind that the minimum and the maximum for the whole development need not be the same as for the initial cleavage stages. Lillie and Knowlton's definition of these terms will be found below.

vestigations have shown (Plough and Ives 1935 and others) that maturation events are specifically altered within the temperature range which yields functional individuals.[3] It is the hope of workers utilizing this method, as has already been emphasized by Needham (1933) and Tyler (1936a and b), that temperature modification may serve to dissociate some of the processes characteristic of development, and thus that the relation between those processes and differentiation may be more clearly envisaged. Efforts in this direction have already met with some success.[4]

THE APPARATUS

The thermostatic assembly used was that demonstrated in the laboratory at the time of the meetings of the American Society of Zoologists in 1933. It consists of a large tank filled with water which is cooled by an electric refrigerating unit and is constantly stirred by a motor. The entire device is kept in a cold room at about 6° C so that a water temperature of between 8° C and 9° C is maintained in the large bath. Eight small aquaria are suspended in the large tank from frames cushioned to prevent vibration. These serve as individual temperature baths, each containing a thermoregulator and heating element and being stirred by a small motor bearing a propeller. The motors are mounted on a frame independent of the aquaria and their suspensions. The eggs were placed in 50 cc. erlenmeyer flasks which were sunk to the necks in the water of the aquaria close to the thermoregulators but not near the heating elements or the walls of the baths. The contents of each of the flasks were agitated at short intervals by medicine droppers which remained standing in the flasks and by means of which the eggs were sampled. Air was also blown through the water by the droppers thus serving to aerate the medium. The temperature of the water in the aquaria fluctuated regularly within narrow limits ($\pm 0.25°$ C) while that in the flasks themselves was shown in several trial runs to maintain a temperature within $\pm 0.05°$ C. Inasmuch as the intervals dealt with were of either 2° or 3°, this was considered sufficiently exact.

With the baths set at three degree intervals it was possible to cover inclusively the ranges from 9° C to 30° C and from 12° C to 33° C. Experiments with both ranges were made as were also several at two-degree intervals between 16° C and 30° C. The range from 12° C to 33° C is wide enough to cover the entire tolerance for *Chaetopterus* while that from 9° C to 30° covers the upper limit for *Arbacia*. The lowest temperature available was limited by the temperature of the water in the surrounding cold bath and hence the actual minimum temperature for *Arbacia* was not reached. A slight modification in the arrangement of the apparatus will permit the exploration of lower temperatures in the future and is essential in experiments with many forms.

MATERIALS

Experiments with the eggs of both *Arbacia* and *Chaetopterus* were made

[3] Many similar instances have been recorded by botanical investigators.
[4] Unpublished results obtained in these laboratories by Mr. R. W. Briggs.

during the summers of 1935 and 1936. Those of the first summer were made by the senior investigator alone; the two of us coöperated in securing the data in the experiments of 1936. All of the data presented below are taken from the 1936 material.

Arbacia.—Experiments were run in Cambridge during July and early August on eggs obtained from animals shipped from the Marine Biological Laboratory[5] at Woods Hole, Massachusetts. Animals were in fresh sea-water in aquaria in the laboratory about three hours after they were shipped and never failed to reach us in excellent condition. The gametes were obtained and the inseminations made in such a way as to ensure a high percentage of membrane elevation without polyspermy. In the experiments reported in detail (Arbacia 2 and 3) 99% membranes were elevated. After insemination the eggs were allowed to stand for from 4 to 5 minutes when they were transferred to the containers in the aquaria. This required in the neighborhood of one minute so that the amount of development prior to transfer varied slightly in the tests. During the first ten minutes numerous observations were made on the eggs remaining in the dish in which the insemination was carried out to ascertain as exactly as possible the condition of the membranes, the percentage of fertilization, and the normality of the eggs. In several cases in which eggs or membranes did not appear normal, wherever less than 95% of the eggs elevated membranes, and wherever polyspermy was present, the results of the experiments are omitted. Records were made of the time elapsing between insemination and the first, the second, and (in three cases) the third cleavages.

Chaetopterus.—Mature adults were obtained from the Marine Biological Laboratory the first week in August and brought to the laboratory at Cambridge in cool water in a thermos jug. In the laboratory they were separated and kept in a cold room in shallow crystallizing dishes, each containing a small amount of sea-water which was changed regularly so that the animals remained in good condition for well over a week. Eggs and sperm were obtained by opening the segments, five being required to yield a sufficient number of eggs for the experiment. The eggs were inseminated as in *Arbacia* and two minutes later were transferred to the flasks, the procedure requiring approximately a minute as mentioned above. Observations on the egg of *Chaetopterus* included the time elapsing between insemination and the first, the second and the third cleavages. In addition, the time at which the first polar body was elevated was recorded and a few observations on the time of second polocyte formation were made. Accurate observations of the times of the polar body elevations are difficult to make in a definite percentage of the eggs; they are more difficult in the case of the second polar body than the first. For this reason such records are considered as interesting though in all probability unreliable.

The records themselves are based on observations of eggs removed from each flask at short intervals. As the time of cleavage approached, samples were taken several times a minute and the time at which 50% of the eggs in a given

[5] We wish to express our appreciation to the members of the collecting staff of the Marine Biological Laboratory and especially to Mr. James McInnis for the care exercised in the expedition of the materials.

field showed the cleavage was taken as the time of that cleavage. The number of the eggs in the field varied but was always greater than fifty. Where time permitted, especially in the first and last part of each run, records were made of nuclear behavior. A single observer cannot make observations on as many as three cleavages in more than four flasks if the percentage of cleaved eggs is to be recorded. This was done in all of the 1936 experiments, which, as has been stated above, are those used here. The 1935 records give only the time at which the first egg was observed to complete the cleavage at the stage sought. They compare very favorably with similar data included in the 1936 protocols. Such figures cannot be used safely in the computation of rate. The data obtained applying as they do to the early stages in development, throw no light on the malformations consistently encountered at high and low temperatures. Such observations were made in many of the experiments but the data are qualitative rather than quantitative and will not be considered here.

EXPERIMENTAL RESULTS

Arbacia punctulata.—Taken as a group the eggs of different *Arbacia* females show a marked consistency in their behavior when reared at different temperatures. This consistency is apparent when the data are plotted either by the method of Krogh or by the method of Arrhenius as employed by Crozier (1924) and his collaborators. There are definite differences in the behavior of the eggs between the temperatures of 15° C and 24° C and above 24° and below 15°. In none of the experiments with *Arbacia* was the temperature sufficiently low to reveal the biological zero and hence the method of Bêlehradek cannot be applied. A comparison of the behaviors of eggs from different females shows differences within these ranges which are relatively small but yet sufficiently large to modify slightly the slopes of the various curves. These individual variations are greatest above 24° C, smaller below 15° C, and least between 15° and 24° C.

The data for five of the experiments on *Arbacia* have been abstracted and are presented in Table 1. The first two cases (Arbacia 2 and 3) included the first three cleavages at temperatures from 12° C to 33° C. The third case (Arbacia 4) includes the first three cleavages at temperatures from 9° C to 30° C. In the fourth and the fifth cases the time of the first and second cleavages is recorded at temperatures from 15° C to 30° C in two-degree intervals. It is evident on examination of the table that for *Arbacia*, the highest temperature permitting any cleavage lies between 30.4° C and 32.7° C. It may also be observed that the time elapsing between the first and the second cleavages is approximately that between the second and third. This is more evident in the graphs which are included and about which more will be said below. In the table, the presence of a + sign indicates a figure appearing in the protocol as nearest the time of 50% cleavage but in reality too low. A − sign indicates that the time is too large. The 528 minutes recorded for the third cleavage at 9.6° C for Arbacia 4, is quite evidently off and the relation between the figure recorded and that which might have been expected on the basis of other experiments is such as to indicate

TABLE 1

Experiment	Cleavage	Temperature in ° C and time in minutes							
Arbacia 2		11.9°	15.5°	18.0°	21.3°	24.7°	26.8°	29.9°	33.3°
	1st	172+	109	77	55	42	39	37	..
	2nd	295	168.5	124	89	69.5	65	62	..
	3rd	384	231−	171.5	123	95	88	84.5	..
Arbacia 3		11.95°	15.2°	18.1°	21.3°	24.15°	26.9°	30.05°	32.7°
	1st	183	110	84.5	59	45	42.5	38	..
	2nd	295	176	133	94	73	69	66	..
	3rd	404	237	177	125	96.5	92	93	..
Arbacia 4		9.6°	12°	15.2°	18.1°	21.3°	24.1°	26.9°	30°
	1st	295	180	110.5	78.5	57.5	46.5	41.3	40
	2nd	486	288	174.5	126	94	72.5	65.5	64
	3rd	528?	399	239	172	130	99	92	89
Arbacia 6		15.4°	18.3°	19.9°	21.9°	24.15°	25.9°	27.9°	30.4°
	1st	116−	76	66	56	49	43.5	41	46
	2nd	171.5	122.5	105.5	89	75	69.5	65	71
Arbacia 7		15.4°	18.1°	19.75°	21.8°	24.5°	25.35°	27.7°	30.1°
	1st	104	75	63	54.5	45	41.8	37	35
	2nd	162	119	100.5	85	70	65	58	58

that the error is the result of miscalculation on the stopwatch (expected time 677 minutes ca.).

Parts of the data relative to three of the cases (see Table 1) have been selected for graphical presentation. In Figure 1 will be found the curves representing the time elapsing between the second and the third cleavages in Arbacia 2 and 3, and the time between the first and the second in Arbacia 6. The coördinates are rate (1/t) and temperature in degrees centigrade (method of Krogh). It is apparent on inspection that the behavior between 15° C and 24° C is different from that above 24° and below 15°. It is also apparent that between these two temperatures the relation is best described, not as a straight line, but as a curve. In Figure 2 may be seen the data for the times elapsing between the first and the second and the second and the third cleavages of Arbacia 2 when plotted as log rate as a function of the reciprocal of the absolute temperature (Arrhenius). Figure 3 is a similar plot of the same data for Arbacia 3. In both, the

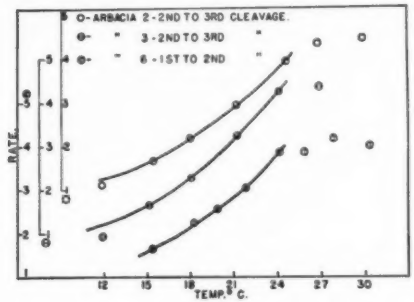

FIG. 1.

difference in behavior between the middle temperature range and the upper is quite evident. Furthermore, the middle range points are best described in terms of a straight line whose slope may be described by its μ. In Table II will be found

TABLE 2

Cleavage interval	Experiment and μ (Temperatures 15° C to 24° C Incl.)				
	Arbacia 2	Arbacia 3	Arbacia 4	Arbacia 6	Arbacia 7
1st to 3rd	16,100	18,000	16,100	..	..
1st to 2nd	16,100	18,200	16,600	16,350	17,450
2nd to 3rd	16,100	18,000	16,600	..	..

Chaetopterus—Insem to 3rd—18,400; 2nd to 3rd—16,100

the μ's for the experiments recorded in Table I. While these vary for the eggs

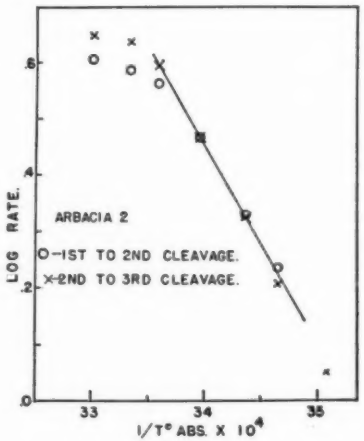

FIG. 2.

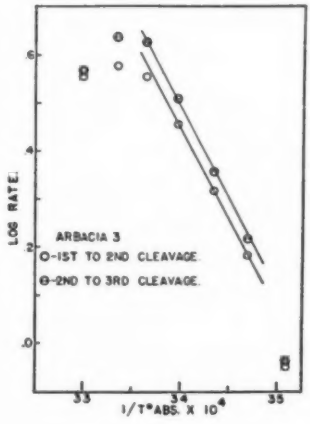

FIG. 3.

of different females, the values for the different periods in the same female are practically the same. Approximate Q_{10} values for the μ's recorded may be found in Table III. The occurrence of such variations between different batches of eggs has been mentioned above. They may be discussed best in relation to considerations of the various parts of the individual curves.

TABLE 3

μ value	Q_{10}
16,100	2.32
16,350	2.35
16,600	2.39
17,450	2.49
18,000	2.6
18,400	2.62

Generally speaking, between the temperatures of 15° C and 24° C the eggs of a single female show a time lapse between the first and second cleavages which is approximately the same as that between the second and the third. Hence the two curves are essentially the same. One exception was found to this rule and is shown in Figure 3 (Arbacia 3). While the time varied it is of interest to note that the way in which rate is effected by temperature does not. Information as to other cases may be found in Table 2.

Relatively little information was obtained on the temperatures below 15° C though that available indicates that the acceleration with increase of temperature over that range is considerably greater than that in the middle range. Such results would be expected on the basis of data assembled by others.

A comparison of the behavior of eggs cleaving at temperatures between 24° C and 30° C with the behavior of eggs at temperatures in the middle range shows differences which may most easily be accounted for by assuming an adaptation. The same sort of behavior is to be seen in the results of Atlas for the frog's egg (1935, p. 308). In *Arbacia* the interval between the first and second cleavages is in every case relatively longer than that between the second and third. It is particularly interesting to note that insofar as those at 24° C are concerned, the time between the first and second cleavages is well below the curve describing the effect of temperature on rate, while the point for the time elapsing between the second and third falls upon the curve. This temperature is particularly useful in understanding the behavior at higher temperatures for, while in each of the latter cases the time from second to third cleavages is shorter than that between the first and second, it is only at 24° C that complete recovery results in a point which falls on the curve for the lower temperatures. Above that temperature the entire curve is shifted. Comparison of Figures 2 and 3 suggests that the adaptation takes place at temperatures below 30° C (Figure 2, 29.9° C) but not at those above (Figure 3, 30.05° C). Whether the adaptation in *Arbacia* would eventually become complete insofar as the processes of cleavage are concerned as is suggested by Atlas's curve (loc. cit.) cannot be determined from the present data. Complete adaptation in respect to cleavage would not necessarily imply typical development. It is quite evident from the present experiments

that there is an initial period of adaptation and that in order to interpret the effects of temperature on cleavage, the investigator must recognize this period and omit it from his time intervals.

CHAETOPTERUS PERGAMENTACEUS

The data obtained from the study of the egg of *Chaetopterus* lead to essentially the same conclusions as those obtained with *Arbacia*. There is a period of adaptation, and subsequently, the rates between 18° C and 24° C show a μ which is similar to that found in Arbacia 2, 4, and 6. The behavior is best described by a straight line in the Arrhenius plot and by a curve in the Krogh plot. The temperatures which permit cleavage, however, are found at the upper part of the range for *Arbacia*, being from 18° C to 33° C inclusive.

Struck by the similarity in the time intervals between insemination and the third cleavage in *Arbacia* and *Chaetopterus*, the data for these two were plotted together and may be seen in Figure 4. In the graph, time is plotted against temperature in degrees centigrade. It may be seen from inspection that the curves are very similar between 18° C and 24° C while below 18° C there is no development (cleavage) in *Chaetopterus* and above 30° C there is none in *Arbacia*. Insofar as cleavage is concerned, the greater effect is to be found at the lower temperatures. The differences may be correlated with the breeding ranges

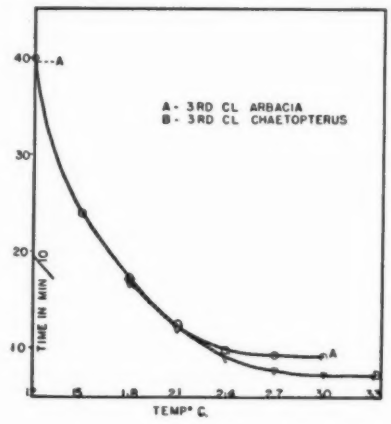

FIG. 4.

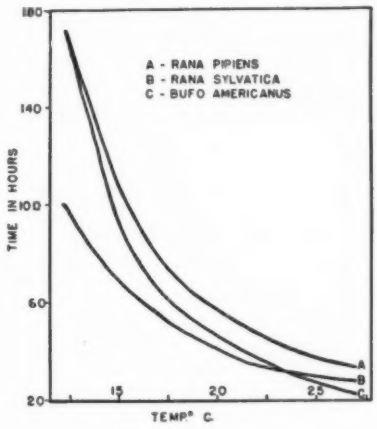

FIG. 5.

of the two forms, a question which will be considered in some detail below in conjunction with as yet unpublished data obtained by Dr. Gertrud Hermes on the behavior of the eggs of several species of Amphibia with different breeding seasons (see Figure 5).

DISCUSSION

Lillie and Knowlton (1897) defined the effects of temperature on regeneration in *Planaria torva*, cleavage and gastrulation in the frog and *Amblystoma*, and

growth of the tail in frog and toad tadpoles in terms of the effects on rate and on form. They state that "the minimum temperature for the whole development is the lowest and the maximum the highest at which normal development may occur." In the above experiments no minimum or maximum temperatures have been determined for the whole development as they have for the early cleavage stages and, as has already been mentioned, it is not necessary that these be the same. Supramaximal temperatures and subminimal temperatures as described by these workers admit of cleavage and hence may be considered as submaximal and supraminimal for those processes. The identification of optimum temperatures is exceedingly difficult and possible only after complete examination of the material for it is more than possible that different optima exist for subordinate events concerned in a single general process. In *Arbacia* it may be suggested that the optimum is at or around 24° or 25° C where the break in the manner in which cleavage is effected by temperature occurs. In *Chaetopterus* the point is less definite but appears to lie somewhere above 27° C. It should be emphasized again that this applies only to the process of cleavage. It should be noted here that the developmental range for *Arbacia*, while extending lower in the temperature scale, shows approximately the same upper limit as that described by Hörstadius (1925) for the summer eggs of *Paracentrotus lividus* and approximately the same optimum temperature.

The above statement applies generally to all of the material studied in spite of the fact that eggs from different females showed slight differences in behavior. These differences are seen in the variations of the μ's and the Q_{10}'s which are given in the tables. This being the case it is evident that in experiments of this kind the more homogeneous the material the more comparable are the results. While previous treatment will undoubtedly prove to be of great importance in explaining the behavior of batches of eggs, genetic differences must also play a rôle. The former may be controlled relatively simply but the latter presents more difficulty.

Hörstadius has demonstrated that the previous history of the adult yielding the gametes has a profound effect on the behavior of the fertilized eggs at different temperatures. In the experiments cited by him the difference in the parents was a seasonal one and hence involved not only the immediately preceding treatment but the conditions under which the oöcytes existed during their growth and maturation phases. In temperature experiments it is impossible to avoid altering the temperature conditions on all but one of the batches of developing eggs. In the experiments cited here, in which gametes were consistently obtained from animals kept at relatively low temperatures, there was evidence of an adaptation which takes place over a recognizable period of time. Evidence for the same kind of thing is contained in Atlas's study of the frog's egg. The information presented above is perhaps more striking inasmuch as it is derived from a study of the earliest periods in the developmental history of the individual and hence the period of adaptation is more closely defined. While there is no positive proof that the adaptation might not be completed at later stages at temperatures above 24° or 25° C this is improbable for the

general shape of the curve for the first to second and the second to third cleavages above that point is not greatly altered.

Both Lillie and Knowlton, and Hörstadius have called attention to the fact that there is a correlation between the breeding season of the species and the behavior of the eggs under various temperature conditions. The observations of Hörstadius are particularly interesting in this respect for they deal with a summer and a winter breeding season of one species, *Paracentrotus lividus*. The winter eggs of this form will develop between the temperatures of 8° C and 23° C while the range for the summer eggs lies between 16° C and 29° C. There is, therefore, a greater discrepancy in the lower temperatures than in the upper. The same effects have been described for several events in the development of a number of amphibians by Lillie and Knowlton. Similarly, in experiments soon to be published, Dr. Gertrud Hermes has found that three species of amphibia, *R. pipiens*, *R. sylvatica*, and *B. americanus*, which have slightly different breeding seasons, also show differences in their responses to various temperature conditions. In Figure 5 may be seen the behavior of these three species (first cleavage to closure of the medullary folds) graphically expressed. Time in hours for the attainment of the stage is plotted against the temperature in degrees centigrade. It is evident that the higher temperatures have a similar though not identical effect on all three while the lower temperature have a greater effect on the development of *R. pipiens* and *B. americanus* than on *R. sylvatica* which breeds earlier in the season. The highest temperature employed (29° ca.) represents the maximum for cleavage for *R. sylvatica*, a slightly supramaximal temperature for the whole development of *R. pipiens*, and permits normal development in *B. americanus*.

A similar difference is to be detected by comparison of the development of *Arbacia* and *Chaetopterus* as expressed in Figure 4 which may be related to the conditions under which the adults live. *Arbacia* occurs regularly at some depth where the temperature of the water is relatively low while *Chaetopterus* occurs in shallow water where the summer temperature is relatively high. The graph shows that *Arbacia* tolerates lower temperatures than *Chaetopterus* i.e. that the minimum temperature for cleavage is much lower, while *Chaetopterus* will complete at least the initial cleavages at temperatures which are above those allowing any cleavage in *Arbacia*. At the temperatures which usually characterize the summer months over the distributional areas of both, the behavior is much the same. These facts must have an effect on the distribution of the species.

A word should be included concerning the graphical method of presenting the data. The methods of Krogh and of Arrhenius which are used above are not intended to have any analytical implications but are used solely for description. The straight line relationship is found in the Arrhenius plot and the curve in that of Krogh as was also the case in Worley's experiments on the development of the fish egg (Worley 1933) and Atlas experiments on the frog's egg. Many others have found the reverse to be true. A somewhat more complete consideration of this question may be found in a paper by Tyler (1936a). More experi-

ments, recognizing the period of adaptation, should be made to discover just which method is more suitable for the description of the data. The method of Bêlehradek could not be used for the biological zero was not determined.

In conclusion it should be stated that the present experiments have demonstrated that there is a period of adaptation on the part of eggs of *Arbacia* and *Chaetopterus* which have been fertilized and are developing at higher temperatures. Moreover, the breeding seasons and the distribution of species in relation to the temperature of the environment are of great importance and influence the response of the developing organism to the altered temperature conditions. The limits and mechanisms of the adaptations should be studied. A series of closely related animals breeding at different seasons[6] or found in different localities should be examined. More extensive information may well aid in the understanding of the general biological question of distribution and the more strictly embryological question of the influence of temperature on the rate of developmental processes.

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[6] The observations of Fage and Legendre, based on the "pêche au feu," certainly indicate that many more invertebrate species have several (perhaps monthly) breeding seasons during the year than has generally been supposed. The lunar periodicity of Nereis and the two breeding periods of *Paracentrotus lividus* (Hörstadius loc. cit.) are well known.

BOOK REVIEWS

FEEDING BEHAVIOR OF INFANTS. Arnold Gesell and Frances L. Ilg. Yale Clinic of Child Development. J. B. Lippincott Co., Philadelphia. 1937. 201 pp. 23 figs. 11 plates. 68 references. Indexed and Cross-indexed.

This volume presents the first systematic account of the growth of feeding behavior from birth through the first years. It stems from the basic point of view that the physiological processes and behavior forms of the organism are expressions of chemical systems and patterned structures: all of which are subject to the laws of growth. The body of the book demonstrates this principle in the feeding behavior of the infant by charts, serial pictures, protocols, and explanatory text. Seven growth periods are designated from the fetal to the second year. The development in each of environmental control through behavior growth is set forth in concise detail. The data are the motor mechanisms, the implements, the techniques, and the behavior of feeding. The evolution from breast and bottle behavior, through cup to spoon behavior, is pictured and correlated with that of expressional, oral, head, and manual behavior growth. The study is a sturdy first chapter in an approach to a developmental hygiene which embraces at once the growing body and mind. As such it is decidedly worth the attention of those interested in growth integration.

F. S. H.

BIOLOGICAL TIME. By P. LeComte du Noüy, with foreword by Alexis Carrel, Macmillan Co., New York. 1937. 180 pp.

Since growth involves the time dimension this discussion of Biological Time will be of interest to all students of growth, not only for the material on different rates of healing of wounds according to age of the organisms but also for the illuminating discussion of time in organisms. The conception of the four dimensional time-space manifold and its implications for biological investigation are presented in a lucid and stimulating fashion that should be of value in making clear the impending conceptual reorganization in biology, with the necessity for abandoning many of the older structural-functional dichotomies and other ideas based upon earlier scientific concepts and beliefs. In this connection attention is invited to an earlier paper by Dr. Carrel on Physiological Time (Science, Vol. 74, No. 1929, p. 620) for further discussion of the difficulties created by the older space *and* time conceptions in biology.

L. K. F.

SOME FUNDAMENTAL ASPECTS OF THE CANCER PROBLEM. Symposium sponsored by the section on medical sciences of the American Association for the Advancement of Science at Atlantic City. Supplement to "Science," vol. 85, 1937.

The papers of the symposium are grouped under the general headings of heredity, stimulation and inhibition of tumorous growths, metabolism of cancerous tissue, radiation, and a general discussion of the problem.

It is particularly gratifying to geneticists to note the advance toward a proper allocation of environmental and hereditary factors concerned with the onset of cancer. The more frequent use of genetically uniform strains of laboratory animals with the consequent elimination of much of the genetic variability so common in older work is of great importance. The constancy of genetic factors in inbred strains of animals

allows the effects of the environmental factors to be allocated more precisely, and in the manipulation of such extrinsic factors lies the great hope of medicine.

We are provided with more knowledge of the chemistry, metabolism and morphology of the cells of malignant tissues contrasted with the conditions of cells in which growth is controlled.

Combined with the attack on cancer waged in the laboratory there is the development of education of all society to regard cancer as a foe which may be conquered if public attention and support are aroused. The reader is convinced that some final answers to the cancer problem should be found sooner because of the coordination of the diverse fields of research through joint publication of representative papers such as these.

S. C. REED

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CONTENTS

Paper		Page
14	MARTIN, G. J. A Calcium Deficiency Syndrome Produced in Growing Animals	175
15	WHITE, P. R. A Comparison of Nutrient Salt Solutions for the Cultivation of Excised Tomato Roots	182
16	HAMMETT, F. S., THEO. LAVINE and M. LAVINE. Developmental Growth and Nucleic Acid Components. V. Cytosine	189
17	REED, S. C. and G. SANDER. Time of Determination of Hair Pigments in the Mouse	194
1	LOEWE, S. and I. W. LOEWE. Intestinal Length and Body Weight in the Adult Albino Mouse	201
19	SIDERIS, C. P. and B. H. KRAUSS. The Growth of Pineapple Plants in Complete Water Cultures with Either Ammonia or Nitrate Salts	204
10	BERRILL, N. J. Cell Number and Organization	211
21	RICHARDS, O. W. and A. J. KAVANAGH. The Course of Population Growth and the Size of Seeding	217
22	HOFFMAN, R. S., J. GOLDSCHMIDT, and L. DOLJANSKI. Comparative Studies on the Growth Capacity of Tissues from Embryonic and Adult Chickens	228
23	HOADLEY, L. and E. R. BRILL. Temperature and the Cleavage Rate of Arbacia and Chaetopterus	234
	Book Reviews	245

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